Characterisation and biological testing of synthetic electrospun membranes for organ-on-a-chip applications

A Thesis Submitted for the Degree of Doctor of Philosophy

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Abstract

Bacterial vaginosis is the most common complex multibacterial vaginal infectious disease among women of childbearing age. It is related to vaginal microecological imbalance and has a high prevalence and recurrence rate. Relevant data shows that the risk of infection is as high as 15% to 50%, the disease may increase a womanIs risk of contracting sexually transmitted infections and lead to premature birth or miscarriage, seriously affecting a womanIs quality of life. There have been few previous studies on bacterial vaginosis systems that mimic the true, in vivo environment incorporating the epithelial layers with bacterial biofilms and the response to treatments. This study developed a two-channel, vagina on a chip that creates an in vitro micro-vaginal tissue that simulates the female lower reproductive tract. The culture chamber contains an electrospun chitosan/polyvinyl alcohol scaffold and vaginal epithelial cells. A diverse attempt was made on electrospinning scaffolds to explore the optimal combination, including comparison of different materials such as chitosan/polyvinyl alcohol, PCL/Gelatin, and exploration of orientation spinning and co-axial spinning. The optimal polymer concentration and composition suitable for the growth of VK2/E6E7 cell lines were explored through MTT assay; fluorescence microscopy verified the possibility that vaginal epithelial cells can adhere to the scaffold; chitosan/polyvinyl alcohol scaffold and vagina on a chip were assembled to construct a 3D in vitro vaginal cell culture platform. The vagina on a chip will allow future clinical applications to explore the causes of bacterial vaginosis and to test related targeted drugs.

keywords: Biomaterials and scaffolds, Chitosan, Organ-on-a-chip, ReproductiVe system, Bacterial Vaginosis.

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Declaration

I, SHUAI QIAO, declare that this thesis characterisation and Biological Testing of Synthetic Electrospun Membranes for Organ-on-a-chip Applications and the data presented in it are original and my own work. I confirm that: this work was done solely while undergoing a doctoral research programme at Brunel University London, UK.

This work has not been previously submitted for a degree at this university or any other academic institution. Attributions to the work of others have been explicitly provided, and quotations from their work have been clearly indicated and appropriately credited to the re-spective authors. In instances where external contributions have played a role in this work, due acknowledgment has been provided, clearly distinguishing such contributions from my efforts.

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List of Abbreviation

2D	Two dimensional
3D	Three dimensional
MTT	3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium
BV	Bacterial Vaginosis
CS	Chitosan
GE	Gelatin
$CaCl_2$	Calcium chloride
DMEM	DMEM/F12 Dulbeccols Modified Eagle Medium/Nutrient Mixture F-12
DMSO	Dimethyl Sulfoxide
Ect1/E6E7	EctocerVical
EGF	Epidermal Growth Factor
EcM	Extracellular Matrix
hcG	Human chorionic gonadotropin
HUVEc	Human umbilical Vein endothelial cells
HIV	Human immunodeficiency Virus
MRKH	Mayer-Rokitansky-Kister-Hauser
OOc	Organ on a chip
PcR	Polymerase chain reaction
PBS	Phosphate Buffer Solution
PDMS	Polydimethylsiloxane
PMMA	Poly(methyl methacrylate)
PGA	Polyglycolic acid
PLA	Polylactic acid
PCL	Polycaprolactone
PLGA	Poly(lactic-co-glycolic acid)
PEG	Polyethylene glycol
PET	Polyethyleneterephthalate

PVA	PolY(vinYl alcohol)
RWV	Rotating wall vessel
SEM	Scanning electron microscope
TEER	Transepithelial-Transendothelial Electrical Resistance
TFE	2, 2, 2-Trifluoroethanol
UV	Ultraviolet
VK2/E6E7	Vaginal Epithelium
VIY	Vaginal hemolysin
Voc	Vagina on a chip

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Chapter 1: Introduction

1. Introduction

The development and progress in the field of tissue engineering have given researchersla greater understanding of different physiological and pathological diseases of the human body. Both in vitro engineered tissue and organ models have been developed based on multidisciplinary technology, allowing scientists to apply them better to for clinical research depending on the specific disease. These different bioengineered organ models can more comprehensively simulate the physiological functions of the human body from molecules to cells, tissues and organs, and even multi-organ combinations of human systems. It includes applications in clinical diagnosis and treatment and drug development, so that researchers can further understand different diseases.

1.1 Bioengineered tissue models

As an in vitro culture platform for studying the pathology of human diseases, biological tissue models mainly use biomimetic human tissues as models to culture cells from different parts of the human body, so as to further conduct biological correlation tests to understand the disease process. The current model systems are mainly based on animal models and two-dimensional cell culture, including pathological studies of different organ parts mainly using experimental mice (sacchi & Bansal, 2020). However, there are huge differences between the body structure of animals and humans, which leads to the fact that the disease research process cannot truly reflect the pathological principles of human organs; secondly, another problem faced by animal models is their high cost and complex operation, which has led to the rapid development of biological tissue models.

Traditional two-dimensional cell culture models usually include culture dishes and culture bottles allow cells to grow in a specific culture environment, so that cells grow in a two-dimensional plane within an extracellular matrix. cell growth is generally divided into two types: adherent and suspended, among which adherent cell growth is the main one. Non-human and traditional two-dimensional culture models have made relatively great breakthroughs in the past, providing a research basis for the pathological process of different human diseases, so that researchers can further understand the pathological changes of the disease. However, the

inherent single layer of the monolayer cell culture system makes the two-dimensional cell culture model unable to simulate the complex function of human organs, and also causes the growth of cells to have certain limitations (Heinrich, 2022). For example, the two-dimensional models lack the interaction between multiple cells or multiple types of cells, which makes it unable to fully reflect the function of human tissues and organs, and thus cannot predict and explain the physiological reactions and drug reactions in the human body.

Based on the in-depth and development of research, the establishment of a three-dimensional cell culture model has greatly increased the feasibility of culturing human cells in vitro and can explain the pathological process of human diseases more comprehensively. It is worth mentioning that most in vitro culture models are based on the culture of human stem cells. Three-dimensional cell culture mainly integrate human cells, cell growth factors and cell growth environment into a platform, so that cells in vitro have the same or similar growth environment as in vivo, effectively simulating the microenvironment of cells in the human body (Doryab & Tas, 2019). For example, the environment in which cells are cultured in three dimensions must be able to truly reflect the growth environment of cells in the human body, including temperature, pH value, nutrients and mechanical properties of muscle tissue. secondly, compared with two-dimensional cell models, three-dimensional cells allow cells to grow and differentiate normally in vitro without serum, which promotes cell growth while ensuring the accuracy of experimental results. Based on the background research, the concept of organ on a chip is proposed to solve the many shortcomings of animal experiments. organ on a chip (OOC) is a specific method that has been used by researchers to produce a more realistic physiological model (chliara & Elezoglou, 2022). OOC has been established using a microfluidic chip that can become a bionic, efficient, energy-saving physiological research and druCg development tool. OOC integrates multiple technologies, allowing different disciplines to cross and jointly create a three-dimensional culture model for the growth of real human cells in vitro. In the field of bioengineering, OOCs are developing rapidly. The in vitro culturable cell platform provides more powerful experimental support for different researchers to study diseases of different organs in the human body, thus enabling them to better

study the pathological process of disease (chliara & Elezoglou, 2022). OOC technology can be used to accurately control multiple system parameters, including chemical concentration gradients, fluid shear forces, construct cell graphic culture, tissue-tissue interfaces and organ-organ interactions (caplin et al, 2015). As a consequence, OOc plays a significant role in the field of biomedicine. At present, the field is still in its infancy, and research on this has just begun around the world, whereas its development has important strategic significance for future human health and the biological industry. As shown in Figure 1, it shows the development history of OOC. From the initial concept to the subsequent development and research of chips of different human body parts, its application in the biological field has gradually become more extensive. For the bioengineering industry, the combination of microfluidic chips and tissue engineering technology makes organ chips more suitable for the study of human diseases.

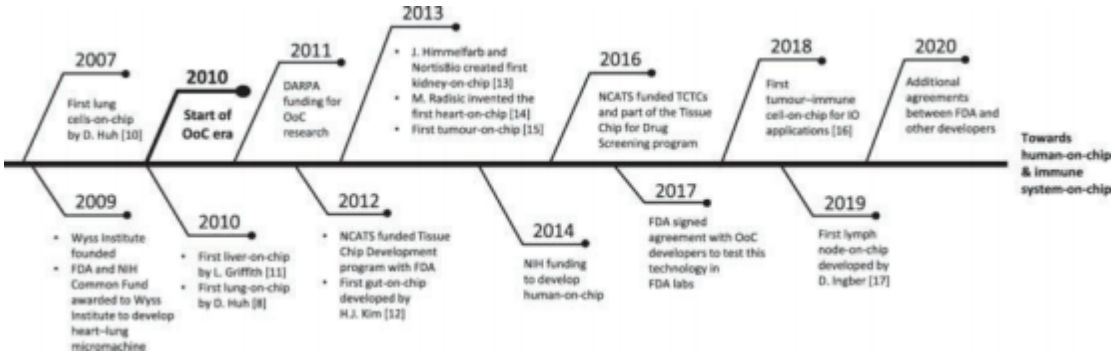

Figure 1.Timeline of the development of OOc. (Clapp, 2021)

1.2 Bacterial vaginosis

1.2.1 Prevalence of bacterial vaginosis

The female reproductive system is a complex and changeable system that includes five different female reproductive parts. Figure 2 shows the structural characteristics of the female reproductive system, which includes four main parts, namely the ovaries, fallopian tubes, uterus and vagina. These different parts have different functions, thus ensuring the perfect operation of the female reproductive system. This figure was drawn by Biorender.com. The vagina is a very important part of this reproductive system. This is the entrance to the female reproductive system. In a normal state it is acidic and Lactobacillus species are dominant. Bacterial vaginosis

occurs when other species become more dominant including Gardnerella and Aptobium. These bacteria cause the vagina to become more alkaline, disrupting homeostasis.

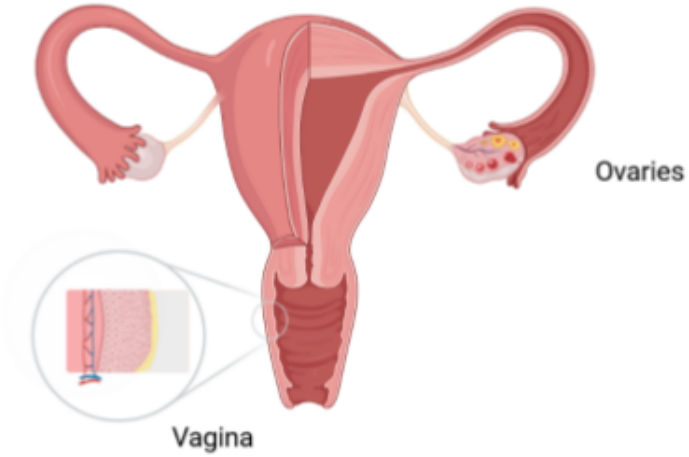

Figure 2. Schematic diagram of the female reproductive system.

According to the 2004 National Health survey Report of the united states (the survey method adopts the commonly used Nugent scoring system), women aged 14 to 49 years old account for the largest group of female BV incidence, accounting for 29.9% (kenyon, Colebunders & Crucitti, 2013). Among these 29.9% women with the disease, half of them show asymptomatic BV; and the BV prevalence rates of non-traditional American women, including Mexicans, black women and Hispanics are 23.2%, 32% and 23% respectively (kenyon, Colebunders & Crucitti, 2013). These non-traditional American women account for the majority of BV patients. In some European countries close to Africa, the incidence of BV in women is as high as 38.5%, including Morocco, Turkey and the Netherlands. The closer to Africa, the higher the rate. The incidence of BV in women in sub-saharan Africa is 40%. It should be noted that the incidence of BV in women who work outside the home is often higher. on the other hand, the incidence of BV in African women is twice that of non-African white women. The lowest incidence of BV in Africa is in the west, such as Burkina Faso, where the incidence is only 7% (Myer,et al, 2005). The incidence in Peru and

Jamaica is 49% among antenatal women and 41% among rural women. BV incidence is low in western European women, including Poland (19%), Norway (24%), and Turkey (23%). BV incidence is also relatively low in New zealand, southeast Asia, south Asia, Indonesia, and Australia, at 30-32% (Jenifer, 2007). Sudan and North America also have low BV incidence, while southern Iran and Egypt have a prevalence of 40% and 33%, respectively. This data shows that the main incidence areas of bacterial vaginosis are concentrated in developing countries, accounting for 50% of the patients, while the proportion of patients in developed countries is concentrated between 20% and 30% (kenyon, Colebunders & Crucitti, 2013). These data show that the risk of women suffering from bacterial vaginosis remains high in different regions. Regional environmental factors and lifestyle factors contribute to a certain proportion of their risk of disease.

However, the prevalence of bacterial vaginosis has regional differences and diagnostic differences. Regional differences are caused by objective factors in developed and poor regions, including the comparison between Europe, The united states, Asia and Africa. As mentioned above, the incidence rate in Asia and Africa is much higher than that in Europe and the united states. The diagnostic differences depend on the differences in diagnostic methods and technologies. The diagnostic methods for bacterial vaginosis are complex and diverse. Differences in diagnostic technologies have led to about 50% of vaginosis being asymptomatic cases (Jack & sobel, 2006). This also indirectly shows that the differences in diagnostic technology make the relevant case data inaccurate. The above description of BV epidemic areas shows that this inflammatory disease is characterized by repeated infection and recurrence. This has also led to a great deal of clinical studies tending to study the pathological mechanism of bacterial vaginosis, thereby benefiting more women.

1.2.2 Predisposing factors for bacterial vaginosis

There are a great deal of factors which can cause bacterial vaginosis, including indirect contact infection, sexual transmission and taking antibiotics in large quantities (coudray & Madhivanan, 2020). For example, in women of normal childbearing age, vaginal epithelial cells proliferate under the action of endocrine hormones and their surface cells are rich in glycogen. The glycogen is conducive to the growth of lactobacillus species which accounts for more than 90% bacteria within

the vagina (Anukam & osazuwa, 2006). The large amount of lactobacillus inhibits the growth of other pathogenic bacteria and forms a normal ecological balance in the vagina.

The bacteria that cause bacterial vaginosis are complex and diverse, including Gardnerella, Atopobium, prevotella, streptococcus and Bacillus (Bautista, 2016). Gardnerella is the most important pathogen causing vaginosis. The vaginal pH for patients with BV changes as discussed before (pH >4.5), causing vaginal Gardnerella to overgrow and produce a large number of amines. This maintains an alkaline vaginal environment and exacerbates the clinical symptoms of bacterial vaginosis (Bautista, 2016). In addition to maintaining an alkaline vaginal environment, vaginal Gardnerella attached to vaginal epithelial cells can release cytotoxins such as vaginal hemolysin and sialidase (Bautista, 2016). Studies have shown that compared with other BV-related pathogens, vaginal Gardnerella shows greater cytotoxicity by producing a cholesterol-dependent pore-forming cytotoxin, vaginal hemolysin (Vly). In addition, the formation of a vaginal biofilm plays a role in the pathogenic process. The initial adhesion of vaginal Gardnerella to vaginal epithelial cells is a key step in the formation of BV and the first step in the formation of vaginal Gardnerella biofilm (Mahajan & Doherty, 2022). In the process of competing with Lactobacillus for adhesion sites, vaginal Gardnerella attaches to vaginal epithelial cells, promoting the formation and continuous development of a biofilm. The presence of oxygen gradients within the biofilm creates a more suitable growth environment for vaginal Gardnerella and improves the resistance of vaginal Gardnerella to mucosal immune defenses (Bradshaw & sobel, 2016).

Bacterial vaginosis is characterized by an increase in the number of anaerobic bacteria in the vagina, and causes harm to women due to the disorder of the vaginal ecology, including susceptibility to sexually transmitted diseases, gonorrhea and human immunodeficiency virus (HIV) infection. On the other hand, studies have shown that bacterial vaginosis is associated with a variety of gynecological diseases, which increases the risk of pelvic inflammatory disease, spontaneous abortion and postpartum endometritis in women (Villaseca & ovalle, et al, 2015). As for BV itself,

up to 50% of BV women relapse within one year after treatment, and the high recurrence rate after treatment can have a serious impact on women's lives.

1.2.3 Detection Methods of bacterial vaginosis

The detection methods for bacterial vaginosis mentioned above are complex and diverse, and the differences in detection methods also lead to the accuracy of case results. Therefore, this section will classify and explain the different detection methods to further understand the mechanism of bacterial vaginosis.

Generally, the detection methods for bacterial vaginosis include dry chemical method, the methods will be described in depth below. The dry chemical method mainly detects pH value using hydrogen peroxide concentration and sialidase (Mohammadzadeh, 2015). For example, if the pH value in the vagina is greater than 4.5, it is considered that the number of lactobacilli in the vagina is lower than the normal value, and the chance of suffering from vaginosis is greatly increased. However, the change of vaginal pH value is variable, including the influence of multiple factors such as flushing, medication and menstruation, which shows that the pH detection method has a large error. The detection of hydrogen peroxide concentration originates from the fact that lactobacilli can secrete hydrogen peroxide to inhibit the growth of other pathogens, and the concentration represents the number of lactobacilli in the vagina (oduyebo, 2009). This is a method of detecting vaginosis by relying on the number of lactobacilli in the vagina, which also has a certain degree of error. The principle of sialidase detection is that Gardnerella can produce this enzyme to fight against the mucous protective factor in the vagina and dissolve vaginal epithelial cells. Therefore, the activity of sialidase in vaginal secretions represents the number of Gardnerella in the vagina. The higher the activity, the more Gardnerella there is and the greater the possibility of vaginosis (Bradshaw, 2005).

The Amsel method is currently the most widely used detection method in clinical practice. It uses four comprehensive detection methods to assess the incidence of vaginosis. They include vaginal secretion pH (greater than 4.5), clue cell positivity (greater than 20%), amine test positivity, and observation of homogeneous thin

gray-white vaginal secretions. The 2021 version of the CDC guidelines in the United states believes that bacterial vaginosis can be diagnosed if the above three conditions are met. This widely used clinical detection method has a specificity of 94% to 99%, but its sensitivity is relatively low, relying on the combination of multiple detection indicators and the operation mode is more complicated (Martins,2023).

The Nugent scoring method is a laboratory testing standard recommended by the Us CDC guidelines. It performs Gram staining on the extracted vaginal secretion smear, and then quantitatively counts the Lactobacillus, Gardnerella, Bacteroides and Mobiluncus. These different bacteria will be converted into a scoring system for evaluation. The full score is ten, 0-3 points are normal, 4-6 points belong to the intermediate type of vaginosis, and 7-10 points can be diagnosed as bacterial vaginosis. This method of staining and scoring vaginal secretions is simpler than the Amsel test and has a certain degree of accuracy (Nugent, 1991). However, the intermediate type of vaginosis with a score of 4-6 is more controversial, and it cannot accurately determine the degree of diagnosis of bacterial vaginosis.

The PCR detection method is to judge the bacteria in the vagina from the molecular dimension, including the relative quantitative detection of the bacterial content in vaginal secretions and the detection of precise bacteria. For example, it can judge vaginosis based on different bacterial references, and the main pathogens that cause vaginal inflammation, including Gardnerella, Mobiluncus, Prevotella and Bacteroides. The large-scale reproduction of this type of bacteria will destroy the vaginal microbial environment, thus leading to the occurrence of BV (GAMa & Mohammed, 2014). The bacteria detected by PCR include different types and affiliations, and the results are more accurate. At the same time, the application of PCR detection kits also makes the detection method of bacterial vaginosis more convenient.

1.2.4 Treatment of bacterial vaginosis

The treatment of bacterial vaginosis is mainly divided into antibiotic therapy and non-antibiotic therapy. Non-antibiotic therapy has greatly reduced the use of antibiotics. which often uses external stimulation or the use of equipment to improve the pH value and the number of probiotics in the vagina.

Metronidazole, Clindamycin and secnidazole are commonly used drugs for the treatment of bacterial vaginosis. They can effectively inhibit the complications of vaginal inflammation and reduce the number of anaerobic bacteria. They are usually divided into two ways: oral and pessary/gel form (Catriona & Bradshaw, 2016). However, the use of metronidazole will inhibit the formation of lactobacilli in the vagina, which means that its dosage is often limited. on the other hand, traditional antibiotic treatment only simply regulates the number of lactobacilli and anaerobic bacteria in the vagina, and it cannot specifically regulate the imbalanced vaginal environment to restore its pH value to normal. Clinical data show that 30% of BV patients relapse within 3 months after a course of medication, and 50% relapse within 6 months (Tomas, 2020). Jack et al. used suppressive treatment to explore the high recurrence rate of BV. He recruited 157 BV patients who relapsed twice within a year, of which 112 women were randomly assigned clinically, that is, 59 patients were treated with metronidazole (using metronidazole vaginal gel twice a week for 16 weeks), and the other 53 patients were treated with placebo for the same length of time, followed by a follow-up visit at week 28. According to statistics, during the entire 28-week follow-up period, the recurrence rate during treatment was 26 (51.0%), while the placebo group was 33 (75%). The probability of maintaining cure with metronidazole was 70%, and the placebo group was 39%, which dropped to 34% and 18% respectively after 28 weeks of follow-up. There are no adverse effects of this procedure; however, the incidence of secondary vaginal candidiasis is higher in women treated with metronidazole. The use of antibiotics also has a certain degree of dependence. once the medication is stopped, it will cause the bacteria to grow again in the vagina, resulting in the recurrence of bacterial vaginosis. In addition, excessive use of antibiotics can lead to a series of side effects, including nausea, vomiting and gastrointestinal damage.

Compared with traditional therapies, the microecological vaginosis treatment method can not only inhibit pathogens, but also improve the microenvironment inside the vagina, reducing medication dependence and recurrence. R. Fluuer (2012) claims that probiotics play an important role in maintaining the health of organisms and can effectively improve the human immune system. On the other hand, probiotics can not only inhibit the growth of related pathogenic bacteria in the

vagina, but also regulate the immune response at the site of inflammation, reducing patients' dependence on medication. Clinical studies (Abbe, 2023) have revealed that vaginosis treatments containing probiotics can effectively reduce patients' Nugent scores. Kingsley collected data from 125 African women in a project evaluating probiotic treatment and traditional treatment for bacterial vaginosis. He used antibiotics and placebos for 60 women, and antibiotics and probiotics for another 65 women. One month after taking the medicine, the two groups of women with different treatments were tested, and the Nugent score and Amsel test were used to evaluate the characteristics of the two groups of women after taking the medicine. The results showed that 88% of women using probiotics and antibiotics had improved inflammation, while only 40% of women using antibiotics and placebos had improved.

In summary, the predisposing factors of bacterial vaginosis are complex and diverse. The imbalance between lactobacilli and pathogenic bacteria caused by changes in the internal microenvironment of the vagina is the main cause of the disease. The detection of vaginosis is currently subject to many restrictions, resulting in inaccurate results (Fan, 2023). For example, differences in regional detection methods, long cycles due to the complexity of the detection methods and detection standards have led to inaccurate patient cases. On the other hand, great progress has been made in the treatment of bacterial vaginosis, including the use of antibacterial drugs and the development of microecological therapy, which enables patients to receive timely and effective treatment. However, recurrence after treatment and drug dependence still cause patients with vaginosis to suffer for a long time.

1.3 Vagina on a chip

The study of bacterial vaginosis is limited by the integration of real human vaginal cells and in vitro culture models, which cannot fully and effectively simulate the real vaginal physiological microenvironment in the human body. However, this research team has developed an in vitro culture model that include different parts of the female reproductive system, including culture platforms such as the vagina, cervix and breast, so that the pathological process and drug delivery of different diseases can be studied more effectively, providing a favorable theoretical and experimental

basis for the in vitro culture of female reproductive systems. The construction of vagina on a chip is a complex process that involves multidisciplinary cross-projects. The use of appropriate materials in bioengineering provides a vaginal growth environment for human vaginal epithelial cells to better support cell growth and differentiation. The traditional two-dimensional cell culture model is limited to its growth pattern and cannot effectively provide an ecological environment for thegrowth of human cells. Therefore, the exploration of building a biological three-dimensional tissue model has become the key to this project.

The vaginal mucosal platform built by Mahajan et al. in 2022 successfully cultivated human vaginal epithelial cells. The platform mainly uses vaginal epithelial cells and uterine fibroblasts to simulate the in vitro vaginal epithelial mucosal structure to explore the in vitro physiological effects of actobacillus and Gardnerella on cells in the vagina. They used a single L. crispatus strain and a combination of multiple L. crispatus Lactobacillus was implanted in the mucosal platform, PCR detected that lactic acid could accumulate on the chip and inhibit the production of anaerobic bacteria; when Gardnerella was implanted in the mucosal platform, it revealed the mechanism of pathogen damage to vaginal epithelial cells. The study successfully demonstrated the physiological interactions between in vitro cultured vaginal epithelial cells and different bacteria and cells. However, it also revealed that most current microbial models cannot fully simulate the characteristics of the human internal microenvironment, including the mechanical properties provided by the peristalsis of muscle tissue, which is essential for the growth of vaginal epithelial cells.

Based on the above description, the vagina on a chip developed and studied in this article focuses on exploring the microenvironment for the growth of vaginal epithelial cells, and combines the theory of bioengineering models to build an environmental platform that is more suitable for the growth of vaginal epithelial cells. Thus, the project combined microfluidic chips and tissue engineering to create one novel organ on a chip which is called vagina on a chip (VoC) to explore the relevant diseases and drug delivery. Firstly, the electrospinning technique is applied into the project to spin the electron membrane to provide a scaffold for the growth of

vaginal epithelial cells. The application of nanostructured scaffolds and biomaterials simulates the mechanical properties of the peristalsis of the muscle tissue inside the vagina, thus providing better mechanical properties for cell growth. Secondly, degradable and biocompatible materials are more suitable for the structure of the human body and are harmless. Then, the VOC needs to be designed using appropriate chips to stimulate the liquid environment of the vagina. This included using PDMS manufactured from 3D printed moulds to provide channels for the flow of culture medium. As a consequence, the designed chips represent the microfluidic aspect to provide micro-channels to form a network with controllable fluid throughout the entire system; the membrane represents the extra cellular membrane that provides the mechanical support to the vaginal cells but will over time, ideally degrade. Finally, the significant procedure of this project is the integration microfluidic chip and electrospun membrane together to culture vaginal cells such as epithelial cell and carry out relevant biological tests.

1.4 Research objectives

The main purpose of this study is to explore a biological scaffold suitable for the growth of vaginal epithelial cells with engineering technology as the core, and to construct a three-dimensional cell growth model with the biological scaffold to combine with microfluidic chip technology to further explore the in vitro growth of human vaginal epithelial cells and the physiological response to binding with pathogens. It can be divided into the following three parts:

① Human vaginal epithelial cells are difficult to culture, and lack the mechanical properties of muscle tissue peristalsis during culture. The three-dimensional culture model can effectively provide mechanical properties for the growth of vaginal epithelial cells to promote their rapid proliferation. This project explores biomaterials suitable for the growth of vaginal epithelial cells, including the concentration ratio of material solutions and related parameters, and uses nanofiber scaffolds made using electrospinning to provide a basis for in vitro culture of vaginal epithelial cells.

② As a powerful platform for in vitro cell culture, microfluidic chips provide a physiological basis for the construction of organ on chips in different parts of the human body. For example, multi-flux design and cross-channels that mimic different organs can provide nutrient circulation for cell growth, thereby simulating the physiological circulation of fluids in human organs. However, due to the different characteristics of different organs in the human body, it is necessary to design chips that are more in line with the specific organ they are trying to replicate. Therefore, the project combined the characteristics of the liquid circulation inside the vagina with the relevant chip design to create a more concise chip liquid channel. The spun three-dimensional biological scaffold and chip were combined to build an overall vagina on a chip and prepare a complete in vitro vaginal epithelial cell culture system.

③ The in vitro culture of vaginal cells and their normal proliferation and differentiation are the basis for subsequent pathogen implantation research. This study conducted routine biological tests on the constructed in vitro vagina on a chip, including cell survival rate analysis to explore the most suitable scaffold for the growth of vaginal epithelial cells. Fluorescence tracking tests explored the structural and morphological characteristics of in vitro proliferated vaginal epithelial cells.

Based on the description of the main objectives of the project, the main theme of this thesis can be divided into four parts, Chapter 2, Chapter 3, Chapter 4 and Chapter 5. Chapter 6 presents the main finding from the thesis.

Chapter 2 is a literature review that describes and analyzes the relevant theories and materials involved in the project. The main technologies evaluated include electrospinning, 3D printing, organ on a chip and in vitro culture of cells. The cross-use of these technologies constitutes the theoretical basis for building a vaginal chip platform. The material properties of chitosan and polyvinyl alcohol polymers were analyzed. Biodegradability and biocompatibility are the basis for in vitro culture of Vk2/E6E7 cells. The polyamino structure in the chitosan molecule enables the material to maintain excellent mechanical properties, which can effectively combine with the hydroxyl groups in the polyvinyl alcohol molecule, thereby improving the spinnability during

electrospinning. At the same time, the application of organ on a chip in the female reproductive system was summarized, revealing that the disadvantages of previous applications were mainly concentrated in the difficult simulation of the microenvironment in the female vagina, and the in vitro culture of vaginal epithelial cells of mice or rabbits lacked authenticity.

Chapter 3 explores the fabrication, characterization performance, mechanical properties and structural components of the chitosan/polyvinyl alcohol scaffolds of four different concentrations of chitosan based on electrospinning technology. The spinning results of the composite material show that the amino group of the polysaccharide structure in the chitosan can effectively combine with the hydroxyl structure in the polyvinyl alcohol molecule, greatly improving the electrospinning effect of the chitosan material itself. The effect of oriented spinning on the mechanical properties of the scaffold was further explored. The characterization results and mechanical tests further show that the arrangement and fiber diameter of the oriented spinning fibers improve the overall performance of the scaffold. FTIR and XRD results show that chitosan/polyvinyl alcohol mixed electrospinning can combine the molecular structures of the two materials, improving spinnability while enhancing the tensile strength and breaking strength of the material.

Chapter 4 explains and elaborates on the design and overall structural features of the vagina on a chip, including the upper and lower layer design patterns of the chip, chip size parameters and assembly methods. The upper and lower layer cross-type single-channel design pattern simplifies the path for external liquid and culture medium flowing through the chip, which can effectively reduce the fluid shear force of the liquid transmitted within the chip, allowing the liquid to be quickly and unimpeded. The method of plasma treatment of the chip surface changes the hydrophobicity of the chip surface, thereby ensuring the stability of the scaffold and chip during assembly, allowing VK2/E6E7 cells to grow and differentiate stably at the scaffold placement, avoiding the growth inhibition effect caused by the instability of the carrier when the cells are cultured in vitro on the scaffold.

Chapter 5 focuses on the biological test after the assembly of the scaffold and chip. An

MTT test was performed on the scaffolds of three different materials to explore the cell growth effect. The culture mode and subculture method of Vk2/E6E7 cells were summarized. The results showed that the oriented fiber scaffold Vk2/E6E7 cells at a concentration of 4% chitosan had the best in vitro growth effect, and the PCL/Gelatin scaffold had the worst effect. The test results confirmed the effect of the mechanical properties of the scaffold material on the in vitro growth of Vk2/E6E7 cells, and further explained that simulating the internal microenvironment of the vagina, including the mechanical effects brought by the peristalsis of muscle tissue is a key factor in the in vitro culture of vaginal cells. On the other hand, the in vitro culture platform after the combination of the vagina on a chip and the Elveflow system was realized. Full integration of the device was realised with cells growing within the chip, adhered to the membrane.

Chapter 6 shows the conclusions made by the author on the whole thesis. This incorporates future work.

Chapter 2: Literature Review

2. Literature Review

This section focusses on the various aspects of research that have been studied by other researchers investigating areas, including bacterial vaginosis, cell culture, microfluidic chips, 3D printing and electrospinning to understand the current research state and development of technology in each field. Based on the introduction to different papers, the literature review aims to give a full and deep understanding of the vagina on chip, it also indicates the therapaputic methods that are relevant the bacterial vaginosis.

2.1 2D Epithelial Model associated with bacterial vaginosis

Female reproductive tract infections are common and frequently occurring diseases in women. Pathogenic microorganisms can cause inflammation of peripheral organs, resulting in diseases such as endometritis, ovarian inflammation, and salpingitis, which are often recurring infections. Inflammation affects the physical and mental health of women. Because the reproductive tract infection may not be detected and treated, which can lead to a series of serious consequences such as infertility, ectopic pregnancy, miscarriage, premature birth and neonatal infections which seriously affect a womenls sexual and reproductive health (kwak et al, 2023). Under normal circumstances, the vaginal microbiota coexists and is in a state of dynamic equilibrium. The balance of the vaginal microenvironment plays an important role in maintaining its self-purification and host health. Reproductive tract surgery, topical medications, vaginal lavage, childbirth, sex life, contraceptives, antibiotics and other factors can perturb the vaginal microenvironment, disrupting the vaginal microecological balance, and cause vaginal microbial imbalance (Doerflinger, 2014).

Human vaginal, ectocervical and endocervical epithelia cells when grown in culture can lead to some differences in gene expression. However studies have showed that the ectocervical and endocervical primary cell lines cultured in vitro, can grow well and the and differentiate normally (Gilbert, 2019). The research on normal epithelial cells cultured in vitro is rare as most studies are focused on cancer. Based on the background, Fichorova (1997) has established an immortalized human vaginal epithelial cell model and passaged the primary cells of the vaginal epithelium. These cells were taken from a 32-year-old woman after vaginal repair surgery; in this research

ectocervical and endocervical samples were taken from different women. These primary cells were immortalized using transduction with retroviral vector (LXSN-16E6E7) packaged by the fibroblast line PA317. These cell lines were shown to form healthy 2D epithelial colonies on subsequent culture and following multiple passages. Figure 3 shows the cultured image of primary vaginal epithelial cells.

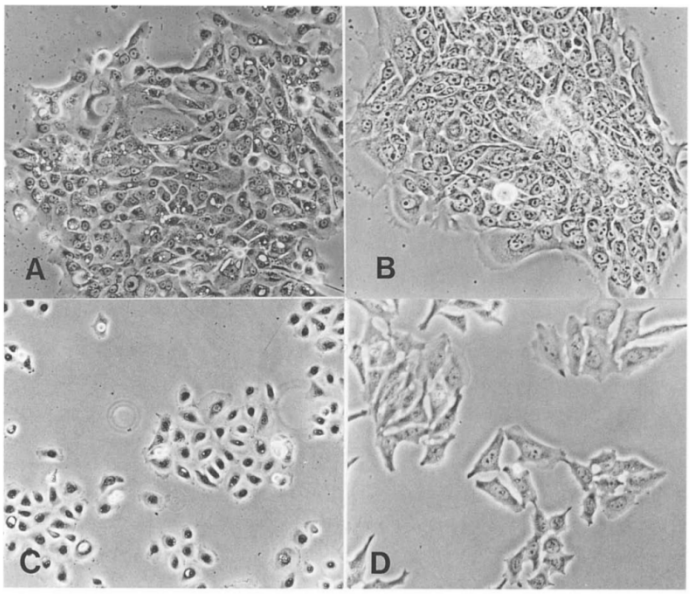

Figure 3. A light microscopy image of immortalized cells (A, EndI/E6E7, B, EctI/E6E7, C, Vk2/E6E7) and of HeLaS3 (D). A-C at x125. D) at x250) (Fichorova, 1997).

The image A/B/C/D in Figure 3 represents the immortalized cell line structure protein structure of all four cell lines Fichorova et al. used; EndI is the endocervix, Ecto, the ectocervix and Vk2 the vaginal line that is used for the remainder of this thesis to grow vaginal cells. As can be seen Fichorova et al. also used the method to transform HeLa cells. In terms of cell passage and differentiation, passage occurs when the cells grow to 90% confluence. Passaged cells can adhere to the wall and appear to 1stretc within 24 hours. After the second passage, the adherence rate is low and the growth 1rest period of 2-10 days is experienced. The cells remain attached to the wall, neither dividing nor falling off to die. The 3rd to 6th generation is the 1golden period of cell growth. The adherence rate of cell passage is significantly increased, and mitotic phenomena are more common during the proliferation period. From the 7th to the 9th generation, the cell volume becomes larger, the shape is irregular, the boundary is fuzzy and small vacuoles appear in the cell, which gradually age and wither, and finally fall off from the bottom of the dish.

Fichorova et al. have established the 2D epithelial model and seeded with bacteria that can be used as a base to model bacterial vaginosis, it has set one successful model to culture epithelial cells in vitro. However, the model which was set up by Fichorova was not optimal, it cannot simulate the 3D structure of the real vagina which means that the culture environment still needs to complete.

Researchers have attempted in vivo models for the vaginal microenvironment with Li et al. (2016) using rabbit and mouse vaginal models to explore the pathological process of bacterial vaginosis. They selected rabbits weighing 2-3 kg and inoculated Escherichia coli and staphylococcus aureus respectively into their vaginas. After three consecutive days of inoculation, the vaginal secretions were extracted to observe the reaction of bacteria and cells. The test results showed that the vaginal mucosa of rabbits had obvious congestion and the surface was protruded by granules. The mouse vagina was implanted with Gardnerella to observe its pathological conditions. The test results showed that the vagina of the mouse was red and swollen and the secretions increased after the bacteria were implanted. The pathological models of these two animals were further investigated and the presence of bacterial vaginosis was characterized by the fast formation, and can well control the type of implanted infectious bacteria. However, the outer layer of the vaginal epithelial tissue of rabbits and mice is quite different from that of humans, and their internal vaginal pH environment is also different from that of humans (Rose, 2012). This demonstrates that although the animal vaginal model can study the pathological process of bacterial vaginosis, there are significant limitations that impact its ability to completely simulate the physiological environment and response of the human vagina.

2.2 3D epithelial cell model

Scientists have found that the physiological state and activity of 2D cultured cells are not completely consistent of those in vivo, and the results often contradict the results of animal experiments and clinical experiments. Based on this background, research has tended towards the development of three-dimensional cell culture to better simulate the human vaginal microenvironment. This allows researchers to more accurately study the pathological process of bacterial vaginosis. In the process of this research, in vitro culturing of vaginal cells is a difficult problem to overcome.

Limited by the growth characteristics of human vaginal epithelial cells and fibroblasts, it is necessary to provide cells with a physiological environment close to that of the human vagina, including the flow of fluids, the peristalsis of muscle tissues, and biochemical reactions (Laniewski, 2017). Therefore, the development of three-dimensional cell models that integrate technologies from more disciplines to simulate the human vaginal microenvironment. Herbst-kralovetz et al. (2020) exploit the 3D epithelial cell models to solve the relevant issues which are associated with bacterial vaginosis. They utilized a rotating wall vessel bioreactor technique to establish a 3D epithelial model to culture the vaginal cells. The rotating wall vessel bioreactor (RWV) allows for a more even distribution of shear forces, thereby reducing heterogeneous shear forces and turbulence in 3D cell culture. The design includes a cylindrical chamber with a gas exchange membrane in which the container body rotates around its axis. However, the scaffold used in RWV is limited in size, the shear stress is still unevenly distributed and it is easy for cells or organoids to collide with the bioreactor wall. On the other hand, Herbest-kralovetz has only seeded vaginal cells, without bacteria associated with BV. Although the 3D epithelial model provides a mechanical environment which was similar to the vagina, the cells culture result was imperfect due to no bacteria was seeded.

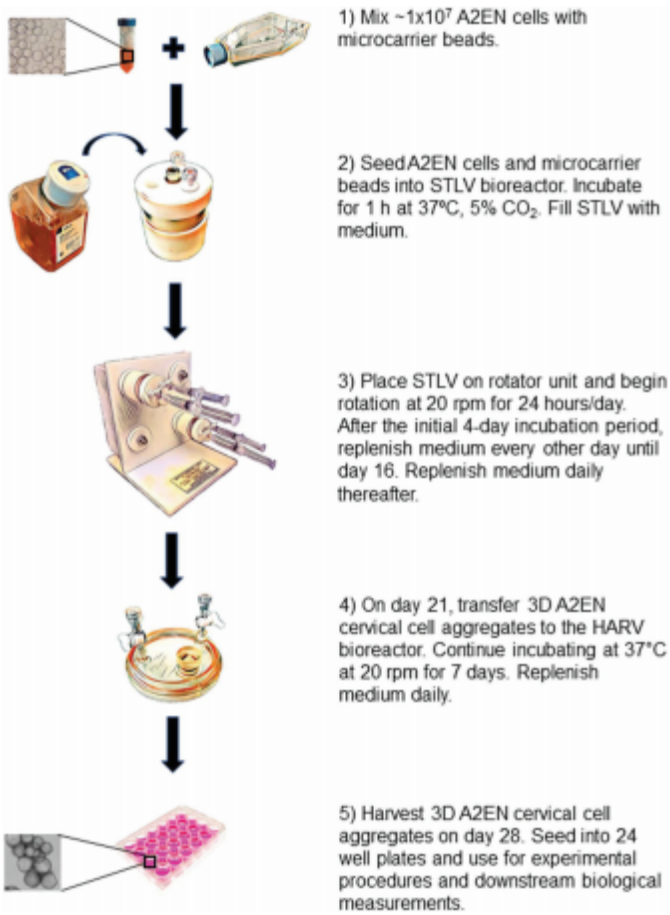

Figure 4. Flowchart of 3D Rotating Cell Culture Model (Herbest-kralovetz, 2021)

Based on the original model, Herbest-kralovetz et al. improved their three-dimensional rotating wall cell culture model. Figure 4 shows the workflow of the three-dimensional rotating wall cell culture model. They embedded a multi-layer inner wall and rectified the culture size to provide more space for cell growth and provide higher shear force to simulate the real microenvironment in the vagina. The key to this model simulating vaginal tissue is to provide a more realistic biochemical environment and mechanical properties for cell growth. The fluid shear force provided by the rotating wall can effectively simulate the mechanical characteristics of muscle tissue peristalsis and promote cell growth and differentiation. The team then implanted cervicovaginal Lactobacillus and Gardnerella into the three-dimensional organoid model to explore the growth of bacteria and cells in the in vitro model, as shown in Figure 5.

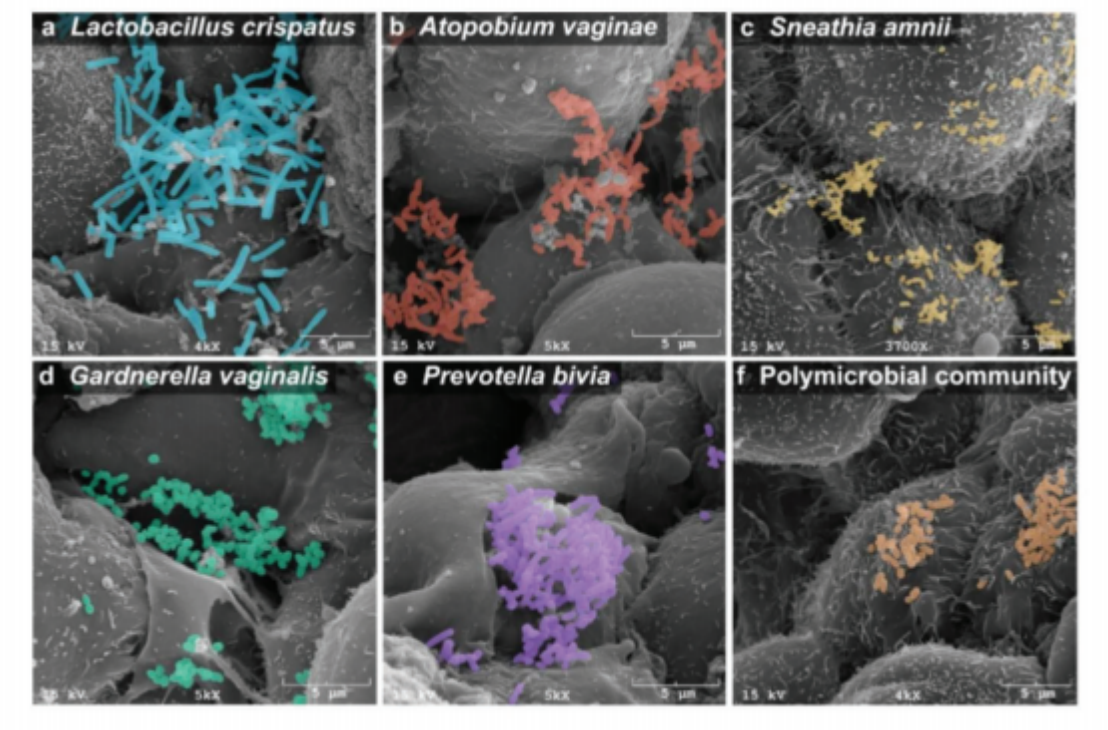

Figure 5. Growth of cervicovaginal Lactobacillus and BVAB in an in vitro 3D cervical epithelial cell model. (Herbest-kralovetz, 2021)

The continuous development and improvement of this model has successfully provided progress for the in vitro culture of cervical epithelial cells, and also demonstrated the feasibility of in vitro culture research of human vaginal epithelial cells.

2.3 Tissue engineering and the engineered vagina

With the development of tissue engineering, its application is becoming more and more extensive with the intersection of disciplines, and the concept is becoming clearer and more familiar. In 1985, Wolter applied the inner cell layer constructed on the Poly(methyl methacrylate) (PMMA) surface to the clinical practice of ophthalmology, and proposed the concept of tissue engineering to explain its application. In 1991, Cima et al. used the bioabsorbable polymer material Polyglycolic acid (PGA) to construct a mesh scaffold to transplant liver and cartilage cells to achieve the purpose of repairing and rebuilding tissues. They also pointed out that the scaffold material should have appropriate surface chemical properties and surface structure, thereby affecting the adhesion and growth of cells; the macroscopic scale of the scaffold, such as porosity has an important influence on the delivery of nutrients. In 1993, Langer systematically proposed the concept and idea of tissue engineering. He explained that a degradable tissue engineering scaffold

with good biocompatibility should be prepared as a cell carrier and extracellular matrix, and then the seeded cells should be planted on the scaffold, and allowed to adhere, proliferate and differentiate on the scaffold. Finally, appropriate growth factors are added to induce the cells to proliferate and differentiate on the scaffold to eventually form new tissues and organs.

The main role of tissue engineering in medical and clinical research is to simulate human internal organs and tissues, thereby providing a more realistic biochemical environment for cell growth in vitro (Edward, 2022). For example, biological scaffolds can provide a more realistic and three-dimensional space for cell growth, allowing cells to grow and differentiate normally. They can also provide certain mechanical properties for special cell growth to ensure the feasibility of cell growth in vitro. Figure 6 shows the basic process of the tissue engineering model: 1. Obtain real human cells that need to be cultured; 2. Culture cells in a culture dish for a certain period of time to maintain normal cell passage to ensure normal cell proliferation and growth; 3. Implant an appropriate number of cells into the prepared biological scaffold; 4. Cultivate in an incubator using special means; 5. Integrate the scaffold for culturing cells (Grabska, 2021).

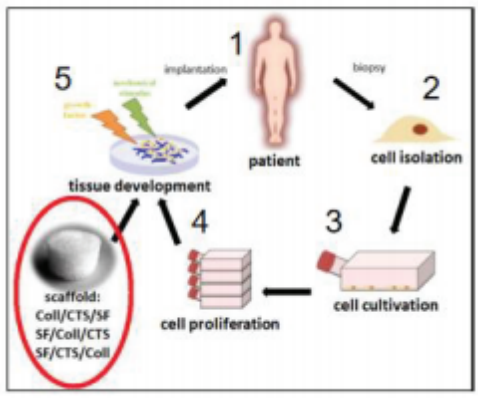

Figure 6 The typical tissue engineering process (Grabska, 2021).

Consequently, most mammalian cells are adherent cells and need to adhere to the scaffold to grow normally. The scaffold provides a place for the growth, reproduction, metabolism and material exchange of seed cells, and can effectively control the growth position of transplanted cells; the scaffold can guide the regeneration of tissues, and its size and shape have an important influence on the structure and

function of tissues. In addition, the scaffold can provide mechanical support for new tissues, withstand external pressure, and maintain the original shape and integrity of tissues (Laniewski & Herbst-kralovetz, 2019). The scaffold can also be used to serve as a carrier of active factors, slowly releasing bioactive substances, such as growth factors, to promote cell growth, proliferation and differentiation. Therefore, the research and development of scaffold materials is the key to tissue engineering. The above description of tissue engineering scaffolds illustrates that different requirements and regulations are required when applied to actual human organs. A good tissue scaffold needs to be able to reflect specific organ characteristics to meet the needs of in vitro cell culture and may differ between different organ types.

2.3.1 Materials for tissue engineering scaffolds

In the biomedical field, the materials commonly used to make scaffolds include four categories: natural polymer materials, synthetic degradable materials, bioactive ceramic materials and composite materials (Eltom, 2019). Natural polymers can be divided into two types based on their structural composition: Polysaccharide natural polymers and polyamine natural polymers. The chemical structure of polysaccharides uses glycosidic bonds to connect different sugar monomers to form a long chain structure; polyamines use peptide bonds to connect different amino acids to form a chain structure (Dobrzanski, 2014). The main advantage of natural polymers is that they contain some biological functional groups that help cells adhere, migrate and proliferate. The disadvantage is that their degradation will inhibit certain functions of cells and it is not easy to control the degradation rate.

The main synthetic biodegradable materials are polylactic acid (PLA), polyglycolate (PGA), and polycaprolactone (PCL). The biocompatibility of this type of material is not as good as that of natural polymer materials, but its mechanical properties are better (kim & Gwak, 2008). Synthetic polymers have two states: semi-crystalline and non-crystalline, and the semi-crystalline polymer network includes unevenly distributed dense molecular chain regions, which mainly plays a cross-linking role and provides mechanical strength for these polymers. The most notable feature of synthetic polymers is that their molecular structure can be changed by chemical bonding or physical mixing through co-polymerization, so they have controllable properties and can meet the needs of different special functions (Dhandayuthapani,

2011). The disadvantage is that their degradation products may have an adverse effect on the matrix, such as the local increase in acidity in the high concentration area of these degradation products. This can cause adverse reactions in different parts of the human body. In addition, high molecular weight polymers are often mixed with other types of polymers to improve their own properties or modify them. However, an inappropriate mixing ratio can also have a negative impact on the original polymer that needs to be modified, so careful preparation is required.

Bioactive ceramic materials such as bioactive glass of silicate and phosphate systems, β-tricalcium phosphate and hydroxyapatite have very slow degradation rates, and some of the degradation products are difficult to absorb (Echeverria, 2021). In addition, the kinetics of ion dissolution and release are still unclear, which makes it difficult to control the release of ions into the physiological environment at the necessary concentration and in the right way. In addition, the main problem is that their mechanical properties have large differences from those of human tissues, such as high elastic modulus and high brittleness, which limits their application (Pennella et al, 2014). Composite materials combine the biocompatibility of natural materials with the excellent mechanical properties of artificial synthetic degradable materials. This type of scaffold is mainly a blend of natural materials and synthetic materials or the use of natural materials to modify the surface of synthetic scaffolds. It is currently a hot topic in the field of tissue engineering. In summary, the choice of tissue engineering scaffold materials is diverse, and they are often classified in more detail according to the actual use scenarios. For example, the different structures of human organs determine the characteristics of the required materials. Due to the characteristics of muscle peristalsis, vaginal tissue often requires biomaterials with better mechanical properties to make scaffolds in order to meet the necessary conditions for cell growth.

2.3.2 Scaffold preparation method

There are many methods for preparing biological scaffolds, but the main principle is to establish a three-dimensional culture structure to promote cell growth and differentiation. Currently, the common methods for preparing scaffolds in tissue engineering are electrospinning, phase separation technology and microsphere method.

2.3.2.1 Microsphere scaffolds

The main method for preparing microsphere scaffolds is to combine a large number of densely packed microspheres by sintering or gel adhesion to form a scaffold with a three-dimensional porous structure (Fu et al, 2018). For example, the scaffold has good connectivity between pores, high mechanical strength, and relatively easy to control the pore size. By using microspheres of different sizes, scaffolds with gradient structures can also be prepared. Kim (2008) et al. used mineralized Poly(lactic-co-glycolic acid) (PLGA) microspheres loaded with BMP-2 to repair cartilage and bone defects in animals by direct injection. Botchwey used high-energy laser beams to sinter PLGA microspheres together. This method has strong controllability and the prepared scaffold has a fully connected network three-dimensional structure. The disadvantage of the microsphere method is that the pore size is small and the porosity is low.

2.3.2.2 Electrospinning scaffolds

The fibrous scaffolds prepared by electrospinning have a high specific surface area and porosity, and the process is relatively simple. However, the scaffolds prepared by electrospinning are usually thin, and multiple layers need to be stacked to achieve a three-dimensional structure. At present, electrospinning technology has been used by a small number of people due to the skill required to produce these fibrous scaffolds. The polymer nanofiber scaffolds manufactured by it are of excellent quality and have been recognized in various fields of tissue engineering (Tuzlakoglu, 2004). There are many types of polymer materials that can be used in electrospinning to achieve the required material properties, and the process of production is relatively convenient.

2.3.2.3 phase separation scaffolds

The phase separation method uses temperature change or solvent composition change to change the solubility of polymer in solvent, so as to make homogeneous polymer solution phase separation. It is usually used in combination with freeze drying to remove residual solvent and obtain porous scaffolds (Liu & Ma, 2009). The scaffold pores obtained by the phase separation method have good connectivity. At the same time, isotropic pores or oriented pore structures can be obtained through different process methods.

2.3.3 Tissue engineered vagina

The traditional clinical treatment of vaginal diseases using non-vaginal tissues to reconstruct the vagina has certain limitations in function. The morphology and histology are quite different from the normal vagina. Complications such as contracture, necrosis, prolapse and intestinal obstruction of the vagina may also occur (Raya-Rivera et al, 2014). Therefore, it is necessary to explore and create a new type of vaginal clinical treatment with less side effects, less trauma that is more in line with physiological requirements. The development of tissue engineering is expected to construct an artificial vagina whose shape and function are close to normal, so as to achieve permanent replacement, perfect shape repair and true functional reconstruction. At present, vaginal epithelial cells and smooth muscle cells are used as seed cells to construct tissue-engineered vagina has achieved initial success. De Filippo (2003) et al. used biopsy of 1cm^2 vaginal tissue from a female rabbit's autologous body and constructed the engineered vagina by in vitro culture and then transplanted it in situ. After 6 months, imaging showed that the vagina was wide and the morphology and physiological function were similar to normal vagina. Panici is equivalent to the vaginal vestibule of a patient with Mayer-Rokitansky-Kister-Hauser (MRKH) syndrome. The whole mucosal layer is collected and the cells are expanded in vitro and used to cover the artificial cavity surface of the patient during vaginoplasty. Observing the length and thickness of the reconstructed vagina and tissue biopsy 1 month after the operation which are all close to normal vagina. Zhu (2021) utilized acellular dermal matrix as a tissue patch to perform artificial vaginoplasty in 5 patients. Follow-up at the 8th week after surgery showed that the full length of the artificial vagina was completely mucosalized. Weng (2013) isolated and cultured human vaginal mucosal stem cells in vitro which successfully constructed a mouse model of artificial vagina using human amniotic membrane as a scaffold. Zhang (2022) et al. inoculated mouse vaginal mucosal epithelial cells cultured in fibrin gel into the inside of pig acellular dermal tubular scaffolds and transplanted them into the vaginally removed mice to establish a tissue-engineered vaginal animal model. It was found that there was complete vaginal epithelium formation 2 weeks after the operation. Observation of samples taken in each period: the length and width of the formed tissue engineered vagina are close to that of normal mouse vagina. According to histomorphological

observation, the surface is stratified squamous epithelium and underneath is the lamina propria containing a large number of collagen fibers and new blood vessels.

2.4 Organ on a chip

Organ-on-a-chip is a micro-cell culture device containing microfluidic chambers and living cells developed based on microfluidic technology. It is a microsystem for organ physiological simulation (Boyang et al, 2018). It simulates the key structures and functional units of human tissues and organs in vitro in a nearly physiological manner, thereby realizing the in vitro culture model of human cells. Generally speaking, the construction of organ-on-a-chip models is based on the understanding and simplification of the anatomical structure and unit functions of specific tissues and organs, so that pathological studies of related diseases can be realized more quickly in vitro (Leung et al, 2022). Based on tissue engineering and microfabrication technology, accurately simulating core factors such as biochemical and biophysical microenvironment, including 3D culture, fluid, shear force, mechanical force, factor gradient, multicellular components, tissue barrier interface and cell-cell/cell-matrix interaction, etc., the specific physiological characteristics of tissues and organs can be reproduced at the organ level, which is difficult to achieve with traditional 2D cell culture systems.

2.4.1 Mechanism of the organ on a chip model

The physiological responses of tissues and organs in the human body are regulated by the coordinated regulation of intrinsic gene expression and external cell microenvironment. The key factors of the cell microenvironment include biochemical factors, such as growth factors, cytokine gradients and nutrients (wu et al, 2020). On the other hand, it includes biophysical factors, such as fluid shear force, mechanical force and electrical signals. These micro environmental factors can guide the occurrence of a series of cell behaviors (such as proliferation, differentiation, migration, etc.) and the assembly and formation of tissues and organs with specific functions. Therefore, this section elaborates on the factors required for the organ on a chip (ooc) formation process and reveals the characteristics that affect these factors.

2.4.1.1 simulating the microenvironment of human organs

(1) Biophysicalfactors

The ooc can precisely control the fluid and fluid shear force by changing the flow rate or channel size. Under the control of the external regulation system, the shear force level of the physiological microenvironment conditions is simulated, thereby improving the distribution of oxygen and nutrients, increasing cell viability and functional maintenance. Studies have shown that fluid and shear force will have a strong impact on cell morphology and function (Ma & peng, 2021). For example, when primary human renal tubular cells are cultured on a chip, physiologically relevant low levels of fluid shear stress promote the differentiation of renal cells, such as polarized distribution of epithelial cells and the formation of primary cilia, which is beneficial to increase the transport function of molecules and drugs and produce toxic reactions similar to those in vivo. On the other hand, the chip can also simulate the complex mechanical microenvironment of tissues. For example, the mechanical strain of muscle tissue can be simulated through a designed PDMS channel, which can also simulate breathing-like air changes, so that cells attached to the porous membrane can be exposed to cyclic mechanical forces and fluid shear forces at the same time, simulating breathing, peristalsis and vascular circulation processes of cells found in the lung (Zhao, 2024).

(2) Biochemicalfactors

Organ on a chip can be used to establish biochemical factor concentration gradients, usually based on fluid splitting and confluence devices to achieve gradient control. This can be used to study cell chemotaxis, stem cell development, or drug screening. It is difficult in traditional cell culture methods to generate regularly changing gradients with precise control. However, this is possible with ooc, for example, Lutolf (2024) reported a gradient chip that can artificially achieve spatial differentiation of stem cells in vitro. The outer fluid channel and the central cell culture channel were separated by perfusing a polyethylene glycol (PEG) hydrogel in the channel to form a barrier, thereby generating a concentration gradient in the central cell channel across this hydrogel. Human pluripotent stem cells were exposed to a central BMP4 signal gradient, thereby achieving spatial regulation of cell differentiation patterns. On the other hand, vascular structures are of great significance for engineered tissues and organs, providing sufficient oxygen and nutrients for tissue growth, and achieving long-term survival and functionalization of tissues. Currently, a variety of chip models with angiogenesis and vascular function

have been created, usually composed of ECM matrix and vascular endothelial cells to form functional vascular channels (Ertl et al, 2014). One model approach uses endothelial cells that sprout or self-assemble in a 3D matrix to form a perfusable capillary network (Bogorad, 2017). Another is a chip containing a network of microchannels with sacrificial materials. The sacrificial material is defined here to allow subsequent cell infusion into the chipIs channels for pre-vascularization. This design approach is widely used in vascularized chip structures, including cardiovascular vessels. After the material is dissolved, endothelial cells are injected into the channels to seed a line them, which can then be used to analyze how spatial gradients affect angiogenesis (Zou, 2020).

2.4.1.2 simulating cell-cell/tissue-tissue interactions

Tissue interfaces are an important means to realize the functions of tissue barriers and the complex interactions between human organs and the physiological microenvironment. Organ on a chip can provide a controllable way to achieve co-culture of different types of cells or tissues, and realize the construction of tissue barriers and biomimetic microenvironments. In other words, the interactions between different cells or tissues in human organs play an important role in the development, homeostasis and disease progression of organs (Kim et al, 2024). Although the culture of a single cell type on an organ chip can simulate some aspects of the tissue microenvironment, it is usually not enough to produce complex structures and functions similar to those of in vivo organs. Organ-on-a-chip technology can simulate cell-cell/tissue-tissue interactions by co-culturing two or more different cell or tissue types in a physiological microenvironment. In addition, the chip design can include multi-layer structures and multiple cell culture chambers. Semi-permeable membranes or porous membranes (such as polycarbonate and PDMs) are often applied to the chip as barrier interfaces. The porous membrane structure is conducive to the permeation and exchange of substances, as well as the analysis of tissue barrier functions and trans-cellular transport, absorption and secretion functions (Barua, 2024). At present, a variety of tissue barriers have been successfully constructed, including an alveolar interface, blood-brain barrier, glomerular-capillary barrier and placental barrier, thereby simulating the key functional characteristics of different tissues. Organ-on-chip-based tissue barriers are

also used to simulate tissue pathophysiological characteristics, study disease mechanisms and test drugs.

In order to construct a bionic human organ micro physiological system in vitro, researchers further used organ on a chip to co-culture different types of tissues/organs, namely multi-organ chips to simulate the interaction of multiple tissues/organs. Multi-organ chips can connect tissue and organ functional modules in different regions through the flow of circulating fluid, simulate blood flow between organs in the human body and promote the exchange, absorption of nutrients and biomolecular signaling. The ultimate form of the multi-organ chip is the construction of a human chip containing all the key organs of the body and therefore can simulate the main physiological functions of the human micro physiological system and monitor the bodyls systemic response to drugs (such as absorption, distribution, metabolism, excretion, etc.). It has great application potential in disease research, personalized medicine and new drug development. As a consequence, with the development of stem cell research, organ on a chip has begun to be used to explore the differentiation, development and disease research of stem cells. utilizing stem cells from the same patient, different tissue and organ models derived from stem cells can be constructed. Combined with multi-organ chip systems, it is expected that personalized human chips will eventually be formed to achieve personalized precision medicine and new drug development.

2.4.1.3 chip sensor and biological scaffold composition

Bio-scaffolds based on chip design can use sensors on the chip to achieve liquid flow and simulate the real physiological response of the human body under the control of the external system. For example, electrochemical biosensors, thermal sensors and semiconductor biosensors can monitor a variety of biological processes and environmental conditions in real time, with the characteristics of sensitivity, speed and accuracy. In other words, organ on a chip can integrate micro sensors to control a great deal of system parameters, thereby transmitting signals to monitor cell culture or micro environmental conditions (Fuchs, 2021).

2.4.2 The development of organ on a chip

2.4.2.1 Lung on a chip

Researchers have exploited Polydimethylsiloxane (PDMS) sheets to simulate the microscopic features of human lungs in layers, so that alveolar respiration and

gas-liquid exchange can be realized (Ingber 2010). This was the first time that an organ on a chip simulated the functionality of a human organ. Figure 7 shows the structure of the lung on a chip. The lung on a chip is combined with two "microfluidics" systems and a synthetic membrane, the membrane is manufactured from PDMS. One system is used for airflow and the other systems is used for culture media. The tracheal epithelial cells and vascular endothelial cells are cultured on both sides of the porous PDMS membrane in the middle, so as to achieve the effect of simulating the air-liquid interface of human alveoli. In addition, in the lung on a chip, the PDMS membrane is pulled on both sides of the circulatory vacuum which means that the cells cultured on it are subjected to a similar lung stretch effect and simulates the effect of alveolar breathing.

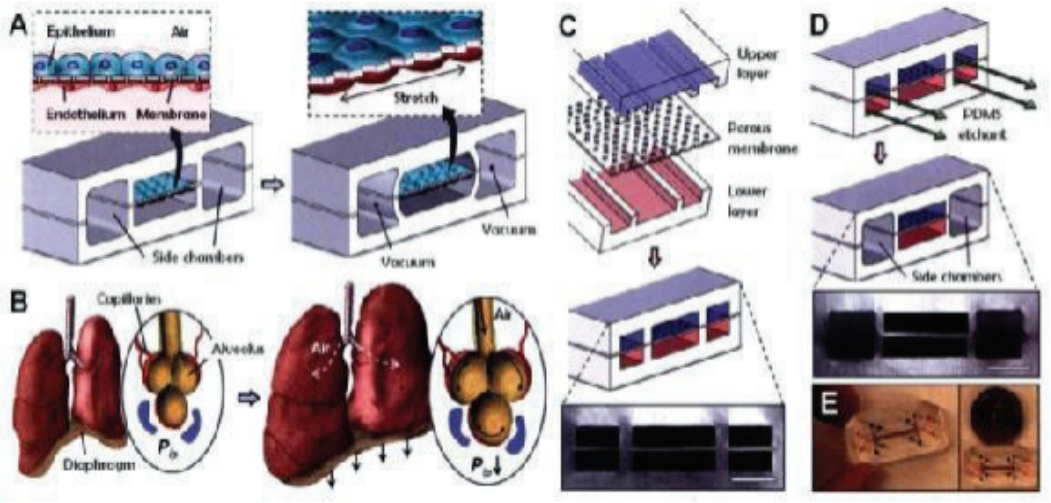

Figure 7. The two-layer lung chip structure created by the Ingber. (Ingber,2010)

2.4.2.2 Intestinal on a chip

Researchers developed an intestine on a chip, which was successful in the formation of intestinal villi in vitro within the human intestine chip to study the differentiation of intestinal tissue in vitro (D. Ingber et al. 2019). Figure 8 shows the intestinal on a chip microdevice. The transparent silicone rubber (PDMS) contains two layers of parallel microchannels(1 mm wide, 10 mm long, and 0.15 mm high). Within the microchannels are caco-2 intestinal epithelial cells, a porous PDMS membrane (10 pores spaced 25 mm apart) that is coated with a layer of extracellular matrix and protein mixture (type I collagen: matrix gel; volume ratio 1:1). This enables cells to

grow normally on the outside of the porous membrane to form a cell monolayer, and the uniform speed of the culture medium also provides conditions for the external growth of cells. The OOC was subjected to 0.02 dyn/cm^2 cyclic mechanical strain (10%, 0.15 Hz) on the straight hollow chambers across both sides of the device to simulate the microenvironment of the mechanical activity of the living intestine, i.e. mimic breathing.

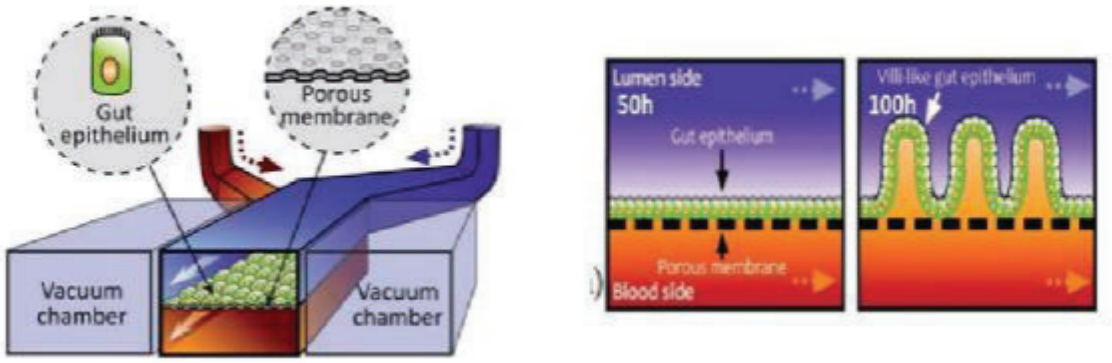

Figure 8. The formation of intestinal villi in vitro on the cultured human intestine chip.(Ingber, 2019)

Intestinal on a chip can also be used for intestinal disease research and drug screening. Beaurivage et al. (2019) used high-throughput intestinal on a chip to simulate inflammatory bowel disease and used it for drug testing. Researchers used cytokine inducers to simulate the effects of Escherichia coli-activated dendritic cells on intestinal epithelial cells. Under inflammatory conditions, the intestinal epithelial barrier function is impaired, resulting in decreased Transepithelial-Transendothelial Electrical Resistance (TEER) values, decreased expression of cell junction proteins, and increased release of proinflammatory cytokines. It was further verified that the anti-inflammatory compound TPcA-1 dose-dependently reduced the release of inflammatory factors in intestinal epithelial cells. This intestinal on a chip model reflects the basic pathological and physiological characteristics of intestinal inflammation and can be used in the detection and drug development of intestinal diseases, so as to more effectively study the pathology of intestinal diseases.

2.4.2.3 Liver on a chip

In 2013, Kang and Sodunke proposed a liver organ on a chip model, Figure 9 shows the mechanism of action of the liver on a chip. They had two configurations, a

single channel configuration which combined a PDMS microchannel and tissue culture dish, or a dual channel configuration, which connected two PDMS microchannels with the microporous membrane in polyethyleneterephthalate (PET). This accurately placed hepatocytes and endothelial cells in layers and co-cultured them in a flowing medium to construct a hepato-like sinusoid model with a specific structure.

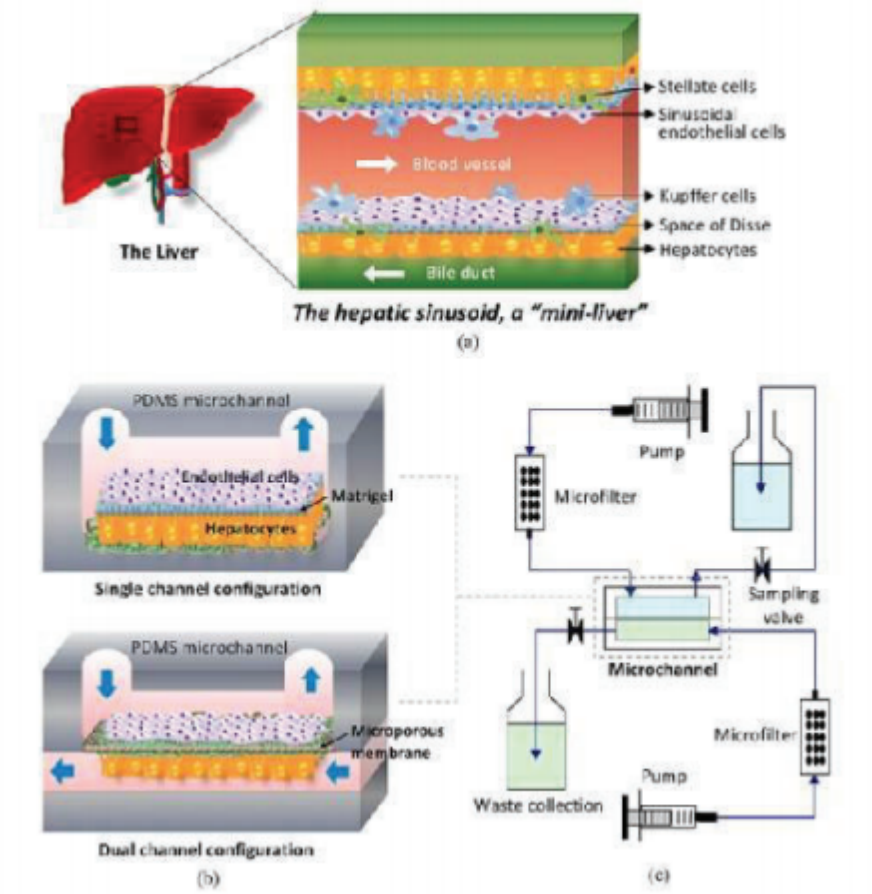

Figure 9. The mechanism of action of the liver chip.(kang & Sodunke, 2013)

Kostadinova et al. (2013) established a co-culture model of hepatocytes and non-parenchymal cells and found that the co-cultured liver model can more reliably reflect drug efficacy and drug-induced hepatotoxicity responses compared with monolayer cultured hepatocytes. Further research established liver chip models from different animal species detected different responses to drug-induced hepatotoxicity, revealing the correlation between hepatotoxicity detected in human and animal experiments, thereby better determining drug safety (Jang, 2019). Rennert (2015) et al. established a perfusable capsular chip to co-culture method, using several non-parenchymal cells with hepatic parenchymal cells. Endothelial cells and macrophages formed the vascular cell layer, stellate cells and hepatocytes formed

the hepatic cell layer, and the hepatic sinusoidal tissue was simulated in a dynamic culture environment. The results showed that perfusion culture can promote the formation of liver microvilli and significantly enhance the function of liver cells. The chip device had also incorporated integrated sensors for realtime detection of cell oxygen consumption. In another approach, Ho (2013) et al. used a radial electric field gradient generated by dielectrophoresis to induce hepatocytes and endothelial cells to arrange in an orderly manner along the electric field direction, simulating the liver lobule structure and effectively increasing the activity of Cyp1A1 enzyme. Deng (2019) et al. used a chip with a concave pit structure to construct micro-liver spheroids in which three types of cells were co-cultured, and then embedded them with chitosan-sodium alginate polymer to form micro-liver tissues that could be transplanted in vivo.

2.4.2.4 Blood-brain barrier organ-on-a-chip

The design of the multi-channel chip can provide the required space and microenvironment conditions for the growth of various types of cells, thereby realizing physiological reactions between various cells and achieving the effect of simulating multiple organs.

The upper and lower structure chips are usually separated by a porous membrane into two channels. The size of the porous membrane is generally less than 10μm, which can intercept cells to prevent the upper cells from falling into the lower layer, and allow the upper and lower cells to contact and exchange substances, and is similar to the cell basement membrane. Different cells can be cultured in the two channels, such as endothelial cells, astrocytes, pericytes, etc. Depending on the cultured cells, the two channels can represent blood vessels and the brain respectively. This structure is somewhat similar to the Transwell system, whereas the structure based on the microfluidic chip is easier to integrate with other devices, such as integrating with a peristaltic pump to provide dynamic stimulation similar to blood flow. This key parameter cannot be reproduced in the two-dimensional static Transwell system. This structure of layering through porous membranes is usually used in chips with blood-brain tissue structures. For example, Shivshankar (2014) et al. designed a similar chip. They maintained the structure of an upper cylindrical channel and changed the lower structure into a rectangular straight channel

separated by an 8μm porous membraneells were grown on top of the porous membrane, and glial cells, neurons and microglia grew on the bottom of the lower channel. As Figure 10 shows, the upper endothelial cells are dynamically stimulated by the connected peristaltic pump to promote the complete formation of the blood-brain barrier. In the improved blood-brain barrier model of the Transwell system mentioned above, the lower glial cells, neurons and microglia are all adhered to the bottom of the channel, and there is almost no direct contact between them and the upper endothelial cells, which is inconsistent with the actual blood-brain barrier structure.

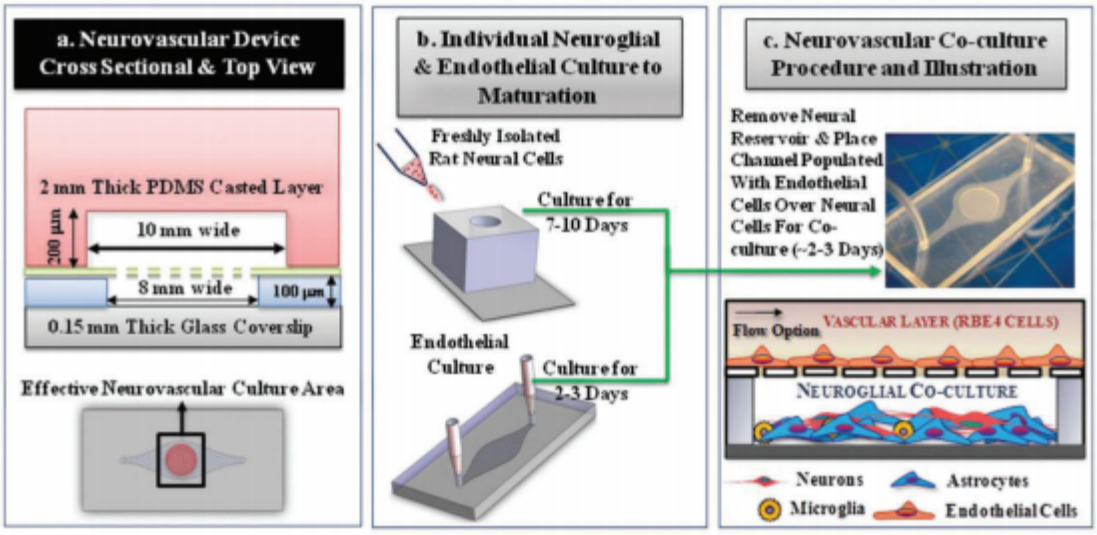

Figure 10. Schematic diagram of the structure of a cylindrical-straight channel microfluidic chip with an upper and lower structure. (shivshankar, 2014)

2.4.3 Chips for womens' health applications

As the various organ on a chip mentioned above, organ on a chip have been applied into the different parts of human and some of these chips have matured, whilst the application into the field of women's health issues has not been researched at the same pace, progress has been shown. iao (2017) et al. used a microfluidic chip system to culture follicles in mouse ovaries for up to 28 days, intending to reproduce the physiological activities of follicles in the 28-day menstrual cycle. compared with static follicle culture, the introduction of dynamic medium can promote the production of steroid hormones in follicles. This microfluidic system simulates the endocrine circuit between the reproductive tract, ovaries, fallopian tubes, uterus,

cervix, and liver organ modules in the female body which continues to circulate between all tissues.

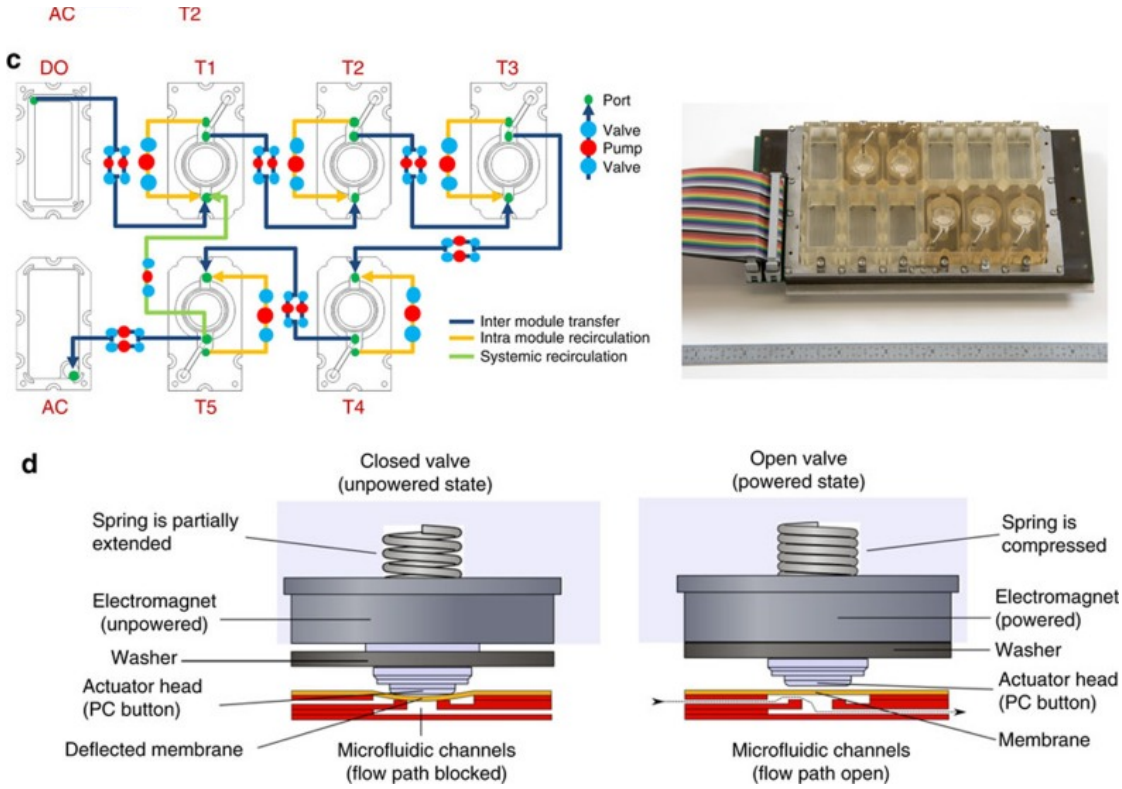

Figure 11. The integrated microfluidic system. (Xiao, 2017)

As Figure 11 shows, the research integrates electromagnetic valves and pump membranes in the microfluidic system and develops a personalized pump program through LabVIEW to control the action of the pump membrane. Continuous application of pressure and vacuum on the electromagnetic valve and pump membrane can produce a peristaltic-like process. Driving the liquid to flow in the microchannel, transporting the fresh culture fluid from the donor to the tissue and removing the old culture fluid and deliver the secreted factors to the recipient which reproduces the communication between the tissues in vitro. Figure 12 indicates the culture progress of the ovarian cells at different stages in this setup. In a single microfluidic module unit, the spatial relationship between the structure of the follicle and the germ cells and their supporting body cells is maintained. In addition, the growth of hair follicles is supported from the primary/early secondary stage to the

sinus stage. After adding chorionic gonadotropin (hcG) stimulation to the single-module microfluidic platform, the ovarian follicle releases mid-term U (MH) oocytes. It can be observed that the oocytes have barrel-shaped bipolar spindles and tightly arranged chromosomes. During ovulation, granulosa cells differentiate into luteal cells after ovulation. These results indicate that a single-module microfluidic platform can support the growth, maturation, ovulation, and luteinization of a single follicle. In other words, the microfluidic chip can be used to cultivate a single human follicle and provides a useful tool for investigating the changes of hormones and their interactions during the process of follicle formation.

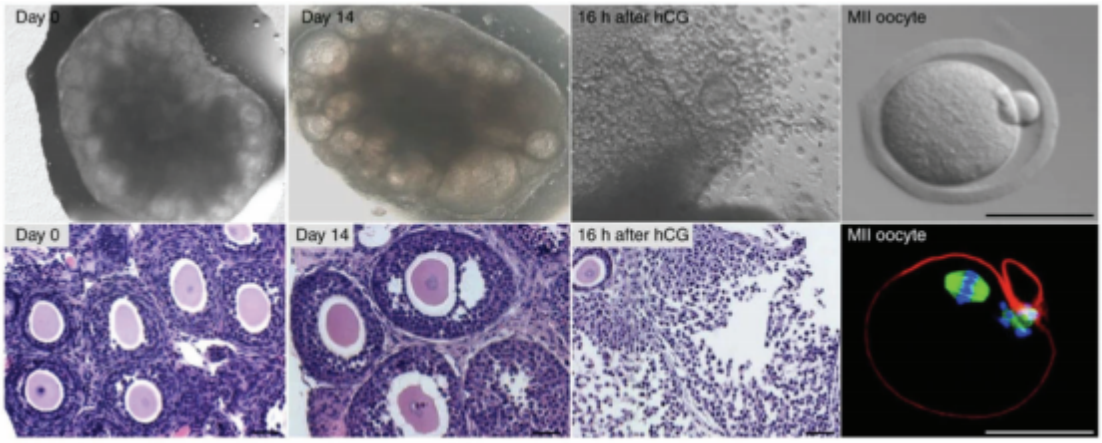

Figure 12. The culture progress of the ovarian cells at different stages. (Xiao, 2017)

Osteen (2019) et al. used the existing microfluidic chip platform to design and develop a dual-chamber microfluidic device to construct a culture system in vitro. As Figure 13 shows, they co-cultured human umbilical vein endothelial cells (HUVEC) with endometrial matrix to construct an in vivo uterine model, avoiding the use of primate models or small animal models with biological uniqueness and less clinical relevance. They tested the function of stromal decidualization by imitating the changes in estrogen and progesterone from proliferation to secretion in the body and observed related phenomena. In addition, the co-culture model can simultaneously analyze stromal decidualization and endometrial vascular functions such as endothelial cellremodeling and vascular barrier formation under controlled physiological conditions, thus verifying the reproducibility of the organ chip model and the ability to check the physiological reproductive process. This is a good model for understanding the complex biological processes and pathogenic factors that affect the function of the endometrium which contributes to screen for agents or environmental toxins that may affect reproductive health.

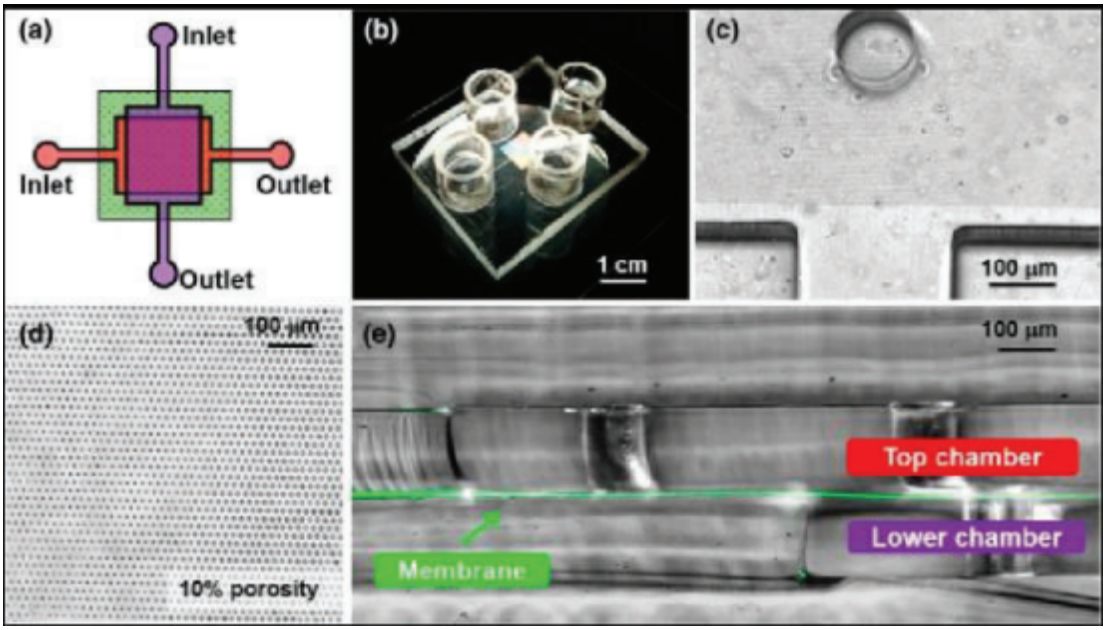

Figure 13. The co-cultured human umbilical vein endothelial cells. (osteen, 2019)

In addition to studying the endometrial matrix, Liu (2022) et al. developed a microfluidic chip based on the artificial uterus to mimic the uterine microenvironment. Embryos are perfused and co-cultured with endometrial cells, and various physiological activities in the female reproductive system or the combination process of reproductive system cells and sperm can be carried out normally on this chip platform. Compared with the traditional petri dish method, the uterine chip based program is easier to operate, and the morula embryo rate and embryo cell rate are higher. Consequently, microfluidic chips which are applied into the female reproductive field is a rapidly developing field. The female reproductive tract contains uterus, fallopian tubes, ovaries, cervix and vagina, the ovary on a chip and uterus on a chip are the researched chips known by the author currently. On the other hand, the entire tissue and tissue-tissue interaction methods are limited, especially in female reproductive organs. Because the ovaries can drive responses to changes in the concentration of hormones secreted by the pituitary gland during preparations for ovulation, fertilization, embryo implantation and placenta formation. The diverse fluid environment, secreting hormones and the complex biological tissue leads the microfluidic application which is used in female reproductive tract to become difficult.

2.4.4 The development of vagina on a chip

Research in vagina on a chip began in Brunel in 2020. The task to produce a vagina on a chip was more complicated than some of the other existing OOc systems, as the

growth of vaginal epithelial cells and the complex microenvironment inside the vagina produced, many unpredictable problems in the construction process of the vaginal chip platform. For example, the growth and proliferation of vaginal epithelial cells are restricted, and the microfluidic chip and the biological scaffold cannot be combined effectively. In 2022, the Harvard Wyss Institute in the united States designed and developed a vaginal chip platform based on PDMS porous membrane as the intermediate medium. As shown in Figure 14, the transmission part of the chip is composed of two layers of intersecting microchannels, and the middle position is a fixed design of PDMS porous membrane for cell growth (Mahajan et al, 2022). The results showed that vaginal epithelial cells and cervical cortical cells were successfully grown on this platform.

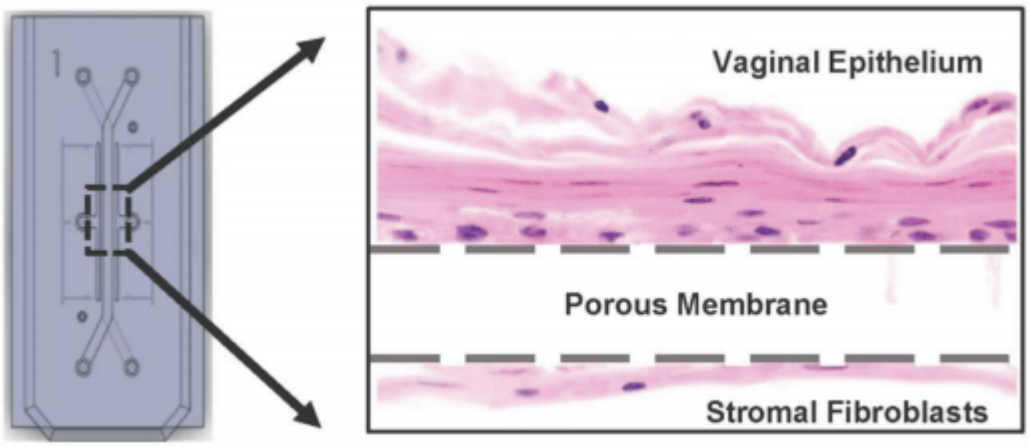

Figure 14. Schematic diagram of the vagina chip platform. (Mahajan et al, 2022)

The Wyss Institute successfully inoculated Lactobacillus and Gardnerella on the platform to explore the biochemical reactions of vaginal epithelial cells and bacteria in vitro. However, the mechanical properties of the porous membrane in the middle of the platform are not sufficient to support the long-term growth and cultivation of vaginal epithelial cells, and it lacks the characteristic function of muscle tissue peristalsis. A six-chamber vagina-cervix-decidua chip was developed to simulate the in vitro culture of multiple tissue cells in the female reproductive system (Ourlad et al, 2022). Figure 15 shows the layout of the chip, which consists of six different chambers composed of PDMS channels to cultivate different cells respectively, and culture medium and nutrients are stored above the chambers.

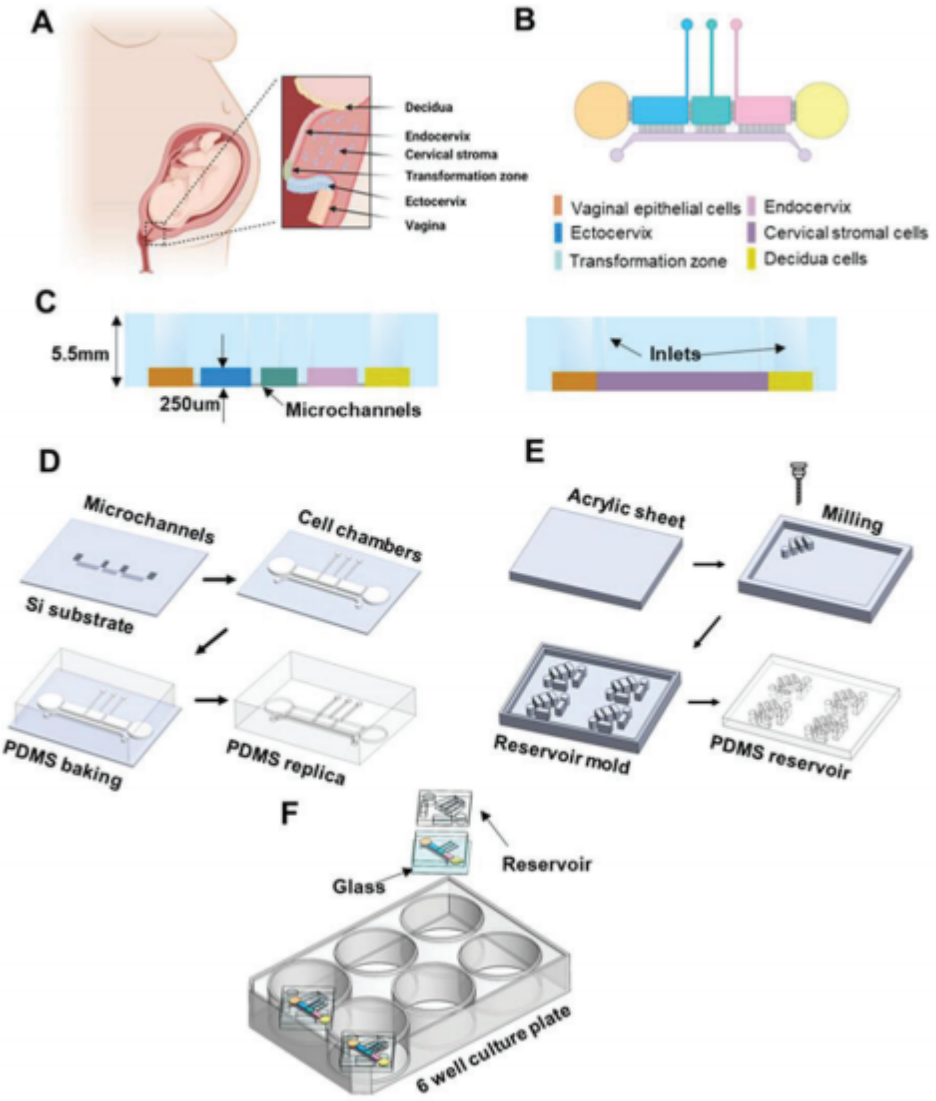

Figure 15. Six-chamber vagina-cervix-decidua organ chip (Ourlad et al, 2022)

The chambers are connected by 24 different microchannels to achieve reactions between multiple cells. This multi-chamber culture of multiple cell types simulates the in vitro culture system of the female reproductive system, but the culture system is two-dimensional and cannot truly represent the diversified expressions in the female reproductive system. For example, the lack of matrix interaction between cells may result in the in vitro culture model being unable to fully simulate real cervical tissue. However, this study provides a basis for in vitro cell culture of the female reproductive system and confirms the feasibility of in vitro culture of female reproductive system cells and bacteria and their interaction.

2.5 Electrospinning

As a major application in tissue engineering, electrospinning technology is widely used in biomedical scaffolds to provide a platform for cell growth. Electrostatic

forces to spin polymers was first used in 1934 (Formhals, 1934). They successfully made the polymer solution eject a fibrous jet under a high voltage. They clearly described the principle of how polymer solution can form a fibrous jet under electrostatic electrodes, and it is considered the beginning of the electrospinning technology for preparing fibers. However, in Formhalsls initial electrospinning experiments, the surface structure of the electrospun fibers was poor and it was difficult to form dry fibers. This was due to the close distance between the transmitter and the receiver. In 1940, he improved the distance between the transmitter and the receiver, thus laied the foundation for the subsequent development of electrospinning (Tucker, 2015).

From the 1930s to the 1980s, electrospinning technology developed slowly. Researchers focused on electrospinning devices and published a series of patents, but it did not attract widespread attention. In the 1990s, the Reneker research group at the university of Akron in the united states conducted in-depth and extensive research on electrospinning processes and applications. The group studied the electrospinning process of a large number of polymers and tried more than 20 polymers using solution or melt electrospinning. The prepared fibers had diameters ranging from 40 nanometers to 2000 nanometers. Since then, electrospinning technology has begun to flourish (Babar et al, 2019).

Compared with other technologies, the preparation of nanomaterials by electrospinning is simple, versatile and low-cost. The high porosity of electrospinning membranes can effectively control the invasion of exogenous microorganisms, and the application of wound dressings is conducive to the exudation of wound fluids (Agarwal, 2013); the electrospinning membrane has a very large specific surface area and can load a large number of active molecules, which is conducive to the encapsulation and release of effective drugs; moreover. Electrospinning technology has strong flexibility in the selection of raw materials, and can develop new fiber materials and applications in large quantities at low cost (Li,2021). Among them, the electrospinning organic/inorganic hybrid nanofiber realizes the composite of two or more components on the nanometer scale, so that while exerting the advantages of each component. As a consequence, the electrospinning technique plays a significant

role in the development of organ on a chip, the membrane which is spun by the electrospinning can simulate the microenvironment of various human organs and improve the relevant properties when cells are cultured on them.

2.5.1 The mechanism of electrospinning

Based on the above development process of electrospinning technology, its application in many fields can be clearly understood. Therefore, this section will explain the technical process of electrospinning, including the operation process and the parameters that affect the results of electrospinning. The main process of electrospinning is that under high voltage drawing, solutions of different polymers are stretched into receivable fibers. As shown in Figure 16, its main components include a high-voltage power supply, a syringe pump, a syringe with a jet needle, and a conductive collector. During the electrospinning process, due to surface tension, the liquid is squeezed out of the needle to produce a hanging drop. The polymer solution under high voltage will change the surface tension of the solution under the action of electrostatic repulsion, so that the solution state changes and is drawn into a continuous Taylor cone shape (Park, 2010). The formation of Taylor cone represents that the polymer solution can be smoothly spun into fibers. This process is not always stable, and it is accompanied by droplet generation or unstable fiber jet. As the solvent evaporates, the jet forms extremely fine fibers that are deposited on the surface of the collector.

Generally speaking, the electrospinning process can be divided into four consecutive steps: (1) The polymer solution is stretched under the action of electrostatics to form a TaYlor cone jet; (2) The fiber jet is extended and ejected in a straight line; (3) The extended jet becomes thinner under a stable electric field, and the jet begins to oscillate; (4) The jet takes on a fibrous form and is ejected to the receiver, thus forming a membrane-like fiber structure.

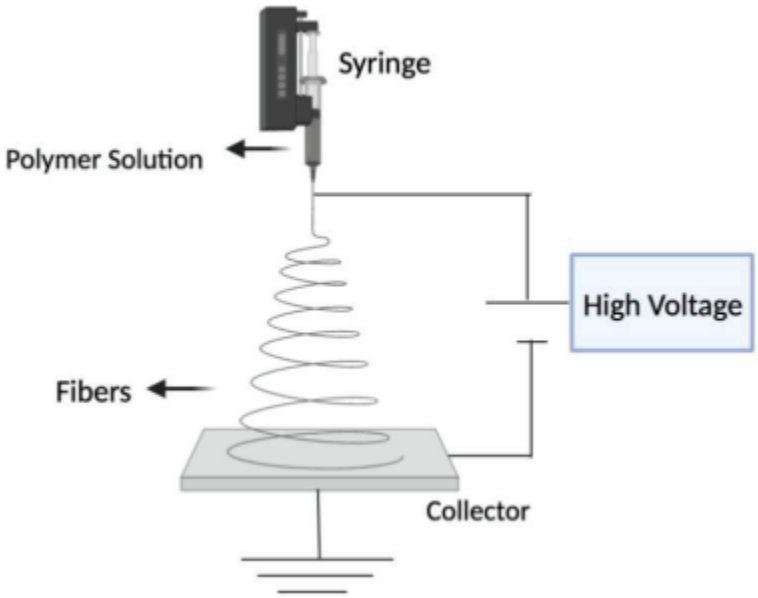

Figure 16. The ordinary process of the electrospinning

Consequently, the research of electrospinning technology applied into the biomedical engineering area has matured based on the unique characteristic of electrospun membrane. For example, in order to achieve the best therapeutic effect in medicine some drugs need to be released slowly into the body tissues, whereas others need to be released quickly. The drug-loaded nanofibers can be used to precisely control the drug release (Angammana, 2016). In addition, the scaffolds made by electrospinning can maximize the characteristics of different materials, allowing these materials to be used in different fields, thus achieving personalized applications. This is also due to the excellent characteristics and controllable operability of the electrospun fibers themselves.

2.5.2 Parameter factors affecting the electrospinning process

During the electrospinning process, polymer fibers will be deposited layer by layer on the receiving device and eventually form a fiber membrane material with a three-dimensional mesh structure. The process of preparing nanofibers by electrospinning will be affected by various factors, which can be divided into the following four categories:

(1) Properties of polymer solution

The properties of different materials themselves determine the structural characteristics of electrospun fibers, thus affecting the application characteristics of

the scaffold. The concentration of spinning solutions prepared with different materials represents the molecular weight of the polymer and directly affects the structural characteristics of electrospun fibers. The molecular weight of the polymer is related to the rheological and electrical properties of the solution, and its size will directly affect the formation of the fiber (Xue, 2019). The concentration of the solution is directly related to the molecular weight of the polymer. When the molecular weight of the solution and other conditions are the same, the concentration of the solution determines the entanglement of the polymer molecular chains in the solution. Due to the different lengths of polymer molecular chains, the viscosity of different polymer solutions at the same concentration is different. The viscosity of the solution directly affects the electrospinning fiberization process (Ji, 2024). Too high a solution viscosity makes it difficult to pull the spinning solution and form fibers; while too low a solution viscosity easily produces droplets, thus affecting the morphological characteristics of the fiber surface.

(2) Properties of the solvent

The properties of the solvent have a certain influence on the formation of uniform ultrafine fibers. During the electrospinning process, the jet is stretched into ultrafine fibers under the action of the electric field force, and then solidified and formed after the solvent evaporates. For solvents with good solubility, high volatility and certain conductivity, the jet is subjected to large traction when the spinning solution is stretched, and it is easy to obtain fibers with finer diameters; otherwise, fibers with beads tend to be obtained.

(3) Spinning process parameters

Spinning process parameters, including spinning voltage, spinning solution flow rate and fiber receiving distance can affect the formation of fibers, whereas these influencing factors are not independent and have a certain correlation with each other. The voltage during the spinning process determines whether the spinning solution can be stably drawn out of the needle tube to form a stable fiber jet (valizadeh & Mussa, 2014). The voltage changes the liquid tension on the surface of the spinning solution, which determines the stability of the liquid during the fiberization process. The flow rate of the spinning solution also has a certain degree of influence on the smooth fiberization. The receiving distance of the fiber affects the electric field strength and the jet flight time. Under the same other conditions,

shortening the fiber receiving distance will increase the electric field strength and shorten the jet flight time, which may cause the solvent in the jet to be deposited on the receiver before it is completely evaporated, and the jet will bond to form a flat fiber.

(4) Environmental parameters

The influence of the environment during electrospinning on fiberization is complex and variable, including parameters such as environmental humidity and temperature. Temperature affects the evaporation degree of the solution during the spinning process, which in turn affects the humidity level of the spinning, affecting the fibers of the spinning jet (Li, 2013). For example, when the temperature rises, the evaporation rate of the spinning solution increases, thereby increasing the humidity in the environment and weakening the stretching effect of the electric field on the solution. Therefore, the influence of the two environmental parameters of temperature and humidity complement each other. The effect of ambient humidity on electrospinning is mainly manifested in the interaction between humidity and the solvent in the jet. This is because during the spinning process, the exchange of solvent with the surrounding air when it evaporates is a double diffusion process. If the ambient humidity is compatible with the solvent, the humidity increases, the solvent evaporation rate slows down, the fibers are easy to stick together, and it is easy to form flat fibers; on the contrary. If the ambient humidity is poorly compatible with the solvent, the increase in humidity will cause the solvent evaporation rate in the jet to increase, the jet solidification time to decrease, the fiber diameter to increase and the distribution to be wider (Ahmed et al, 2015).

2.5.3.Oriented Electrospun nanofibers

Oriented electrospun nanofibers are an improvement on nanofiber based on electrospinning. They can increase the strength of nanofibers by arranging the fibers with an orientation degree, thereby providing better mechanical properties to the scaffold. Common preparation methods for oriented nanofibers include electrospinning using a roller receiving device, electrospinning assisted by an electric field or magnetic field, and near-field electrospinning (Baji, 2010). Among them, ordinary roller receiving devices are widely used to prepare oriented electrospun fibers. During the electrospinning process, the pulling action of the rotating receiving device offsets the whip flow of the fiber, so that oriented fibers can be formed on the

roller. This method can produce continuous and highly oriented micro-nano fibers, and its production scale is large and efficient. However, it has high requirements for the rotation speed of the roller (usually 1000rpm and above), which may cause certain safety issues.

Figure 17 shows the principle process of oriented electrospinning roller. Its principle is similar to that of ordinary electrospinning, but the receiving device becomes a roller with a constant speed. The nanofibers are ejected from the needle to the roller device with a constant speed by voltage, and then formed into an arrangement with a certain degree of orientation under the drive of the roller (Wang, 2020). The electron microscope image shown below shows the degree of arrangement of the oriented fibers, which clearly shows that the orientation of the fibers is more regular and the diameter of the fibers is relatively thin.

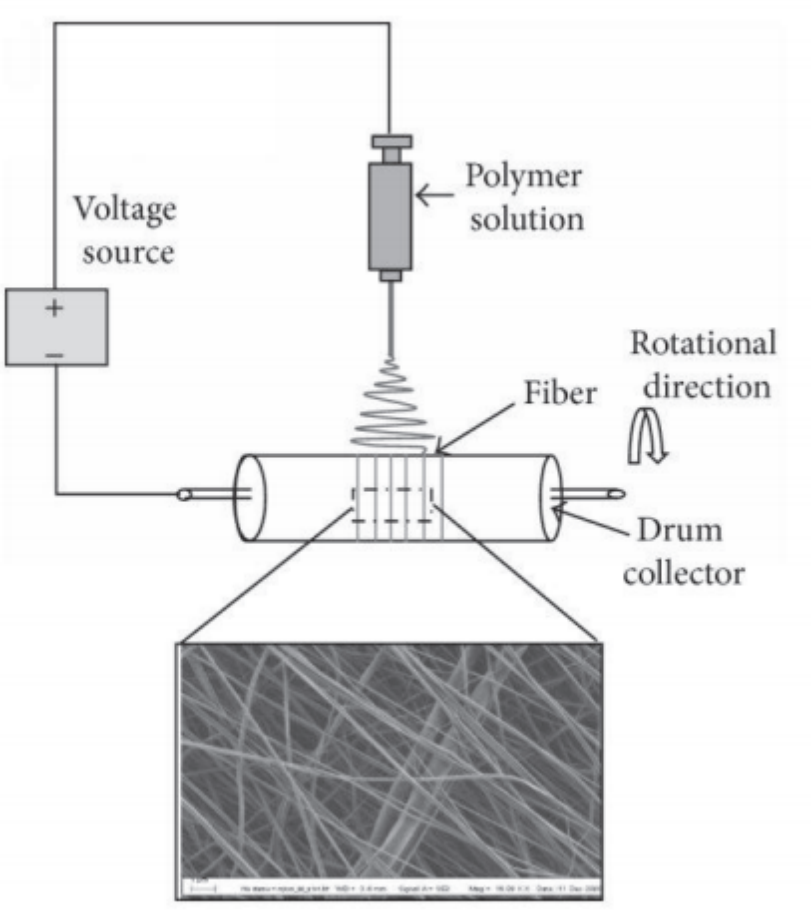

Figure 17. Principle and process of oriented electrospinning.(Wang, 2020)

The application of oriented fibers in bioengineering is diverse, which also highlights that the bioscaffolds prepared by them have good mechanical properties to provide cells with a better growth environment. Wang (2020) et al. prepared a double-layer electrospun membrane with an oriented fiber layer inside to promote tendon healing and a random fiber layer outside to prevent adhesion around the tendon. This innovative design can effectively prevent adhesion around the tendon and improve the quality of tendon healing. Oriented nanofiber scaffolds can provide morphological clues for seed cells to guide cell directional growth. Adding certain bioactive factors to oriented nanofibers can play a synergistic role. Chen (2013) et al. designed a "core-sheath" structure nanofiber membrane with PCL as a biochemical clue and fiber orientation as a topological morphology regulator. The combined action of the two promoted cell proliferation and upregulated the expression of tendon-related genes and proteins. studies have shown that compared with oriented fibers, disordered fiber morphology is more likely to induce pro-inflammatory expression in macrophages, tendon fibroblasts and tendon tissue. This interaction between matrix morphology and immune cells provides

2.5.4 Research status of Electrospinning Nanofiber Membrane in Cell Culture

The electrospun nanofiber membrane is breathable and moisture permeable which means that it can fully absorb secretions and is highly compatible with biological tissues. In recent years, the research of electrospun nanofibers has increasingly tended to be used as biodegradable internal dressings and degradable bandages, without causing side effects such as infection. Beyond that, culturing cell on electronspun membrane is the preferable option on the biomedical field. Zhou (2012) et al. used electrospinning technology to prepare a PLGA nanofiber membrane containing mesoporous silica as a scaffold material for bone tissue support. As Figure 18 shows, the content of mesoporous silica is 2.5%, 5% and 10% respectively. Studies have shown that as the content of mesoporous silica increases, the porosity of the nanofiber membrane prepared by electrospinning gradually decreases and the fiber diameter gradually becomes thinner. The above nanofiber membrane was used for the in vitro culture of mesenchymal stem cells, and it was found that the nanofiber membrane added with mesoporous silica as a bone tissue scaffold has a higher cell

survival rate than pure PLGA nanofiber membrane. The higher cell survival rate in mesoporous dioxide. when the silicon content is 10%, the cell viability is the best. The biological activity of mesoporous silica promotes the differentiation and activity of mesenchymal stem cells, thus enabling the cells to grow smoothly on the electrospun membrane.

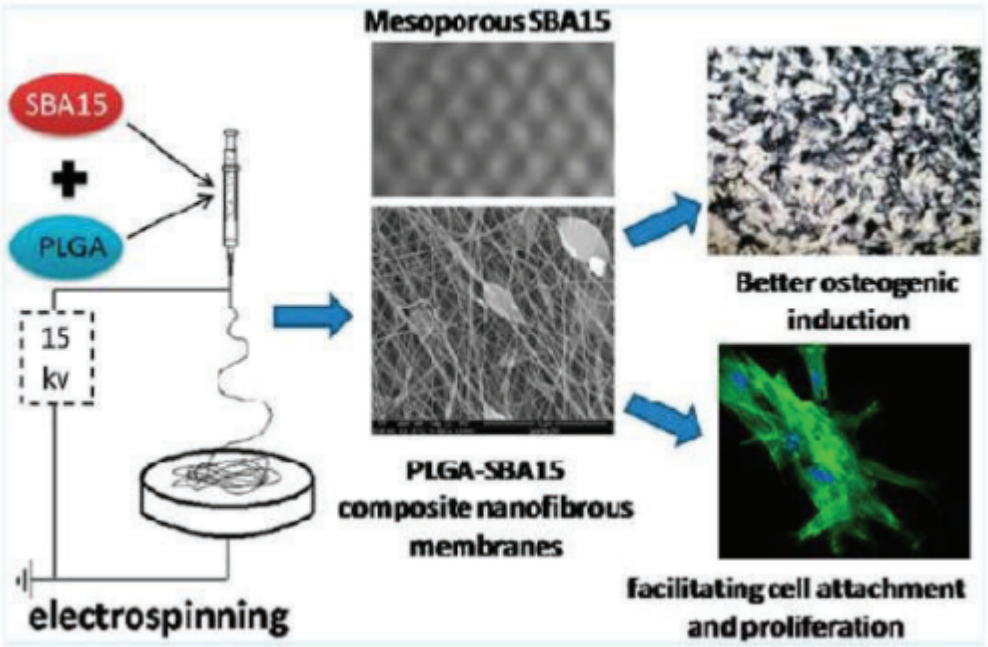

Figure 18. The PLGA nanofiber membrane. (Zhou, 2012)

On the other hand, yang (2016) et al. used electrospinning technology to prepare PLGA nanofiber membranes rich in hydroxyapatite and collagen for the three-dimensional culture of bone marrow mesenchymal stem cells in vitro. Studies have shown that compared with pure PLGA nanofiber membranes, the contact angle of PLGA nanofiber membranes compounded with hydroxyapatite and collagen gradually decreases, indicating that the addition of hydroxyapatite and collagen increases the hydrophilicity of nanofiber membranes. The original hydrophobic state becomes more hydrophilic. The experiment of culturing bone marrow mesenchymal stem cells on the surface of nanofiber membranes shows that bone marrow mesenchymal stem cells grow and differentiate better on the surface of PLGA nanofiber membranes containing hydroxyapatite and collagen.

As a consequence, the biomaterial which utilizes to spin the membrane, including poly(lactic-co-glycolic acid), polycaprolactone(PCL) and polyvinylalcohol (PVA) are

commonly applied into the organ on a chip to simulate the tissue,s environment. The excellent biodegradability and biocompatibility of these materials provide similar structure to the human tissues and the nontoxic property makes the electrospun membrane harmless to human health.

2.5.5 Application research of electrospinning scaffolds

The diameter of the biological scaffold made by electrospinning is at the nanometer level, and the porous surface can form a three-dimensional structure, thus providing a strong condition for the growth of cells on the scaffold. At present, the polymer ultrafine fibers prepared by electrospinning have been widely used as bone tissue, cartilage, tendon, nerve, blood vessel, skin and bladder scaffolds. Abbasi (2020) et al. stated the effect of scaffold pore size on bone cell adsorption, proliferation and migration. The results showed that small-pore scaffolds have a large surface area, which is conducive to cell adhesion in the initial stage, while large-pore scaffolds can successfully promote cell migration and growth, greatly reducing the degree of cell aggregation. Phipps (2011) compared three methods to increase the pore size of polycaprolactone/collagen/hydroxyapatite (PCL/col/HA) bone tissue engineering scaffolds. They believed that adding polyethylene oxide (PEO) during the electrospinning process can effectively increase the pore size of the scaffold. Bai (2020) studied the biocompatibility of electrospun PLA/PCL nanofibers with different ratios and periodontal ligament fibroblasts (PDLF) and concluded that the fiber scaffold had better compatibility with PDLF when the mass ratio of PLA/PCL was 50/50. In vascular tissue engineering applications, it is usually necessary to inoculate specific cells or encapsulate cells in biodegradable synthetic material scaffolds to induce extracellular matrix. The vascular tissue in the human body is divided into three layers, and each layer adsorbs different cells. Therefore, in order to better simulate vascular tissue and achieve the adsorption requirements of different cells, it is necessary to prepare a multi-layered scaffold. Vaz (2005) used a multilayer electrospinning device equipped with a round roller receiver to prepare a double-layer PLA/PCL tubular scaffold. The results showed that the scaffold can effectively improve cell adhesion, proliferation and differentiation, and promote cell migration and growth. Liu (2015) used mouse adipose stem cells to culture experiments on fiber scaffolds made of different ratios of PLA-PCL/Poloxamer, andfinally obtained a large number of mouse adipose stem cells with complete

cell structure and smooth surface, which has guiding significance for the application of electrospun fiber membrane scaffolds in skin tissue engineering.

In summary, the bioscaffolds prepared by electrospinning of different materials are designed to meet the characteristics of different parts and organs of the human body. One of the notable features of the scaffolds prepared by electrospinning is that they have good mechanical properties. In other words, mechanical action is the most direct and fundamental role of nanofiber scaffold materials in their applications. Both the microstructure and macroscopic state of the fiber membrane will have an impact on cell culture. The mechanical properties of the electrospun membrane used in biological tissue engineering scaffolds are the key factors for cell growth. Related studies have shown that the mechanical properties provided by the scaffold material can effectively promote cell growth and differentiation, thereby improving cell survival. For example, the mechanical properties of the scaffold can simulate the characteristics of muscle tissue and provide a good physiological microenvironment for cell peristalsis, growth and convergence. The toughness and strength of the electrospun scaffold also increase the adhesion of cells, providing a feasible basis for the in vitro growth of cells. On the other hand, the mechanical properties can also provide a certain ductility and elasticity to the biological scaffold, making it easy to operate to simulate the morphological characteristics of different organs of the human body. Beyond that, the mechanical properties of nanofiber scaffolds are determined by the properties of the single fibers that make up the scaffold material and the microstructure of the scaffold. In theory, if we can obtain highly oriented and highly crystallized nanofibers, the nanofiber scaffolds will have higher mechanical properties. However, in existing electrospinning, the spinning path is very short and the stretching rate is low, so the polymer orientation is not well developed. Although researchers have made various designs on the electrospinning receiving device (such as high-speed rotating cylinders, discs, cross-shaped electrodes, parallel circles, etc.), to a certain extent, directional fibers can be obtained to improve the tensile strength of the fiber membrane, but as far as single fibers are concerned, the effect of improving the mechanical properties of the fibers is not significant. However, the composite effect of material properties can be achieved through material

blending, thereby improving the mechanical properties of a single material during spinning.

2.6 3D printing

3D printing is a rapid prototyping technology that is based on digital model files and uses adhesive materials, including powdered metals or plastics to construct three-dimensional objects with complex structures through layer-by-layer printing and layer-by-layer curing. Generally, the model is the most basic element in 3D printing which can be obtained by 3D scanning physical objects or self-modeling software, including 3Dmax, Maya, Proe and Solidworks (Shahrubudin, 2019). Therefore, the developed 3D printing technology have been utilized in a number of fields for the direct manufacture of some products, it has also become an effective means of manufacturing modern model molds and parts. 3D printing provides huge convenience to the manufacture process of the organ on a chip. The impact of 3D printing on organ on a chip is reflected in two aspects, chip devices and organ simulations. The chip device includes not only the chip itself, but also biosensors for online detection. The chip template can be obtained by ordinary 3D printing technology which without cumbersome processes such as cleaning the substrate, spinning glue, exposing and developing, it simplifies the chip manufacturing process (Gopinathan, 2018). In particular, it can produce templates with irregular and special structures. In addition, 3D printing PLA, transparent resin and other materials can obtain microfluidic chips containing microchannels and culture cells for cell or tissue culture.

At present, photolithography technology is mostly used for the production of organ on a chip templates. The basic process includes pretreatment, photoresist coating, pre-baking, exposure, development and inspection and film hardening. However, the multi-step process of this process requires manual operation which is expensive, time-consuming and many manual errors. 3D printing the chip template can save multiple steps, reduce the error of human operation and greatly save the time of making chip template. Figure 19 indicates the 3D printing of chip template. Daniel Filippini (2018) et al. used a 3D printer to print layer by layer to obtain a special resin template. After surface treatment with ethanol, PDMS was poured and oven cured to obtain a PDMS chip. . This template achieves high integration of two different levels

of microns and millimeters which can directly connect the tubing to PDMS to improve its tightness and avoid leakage.

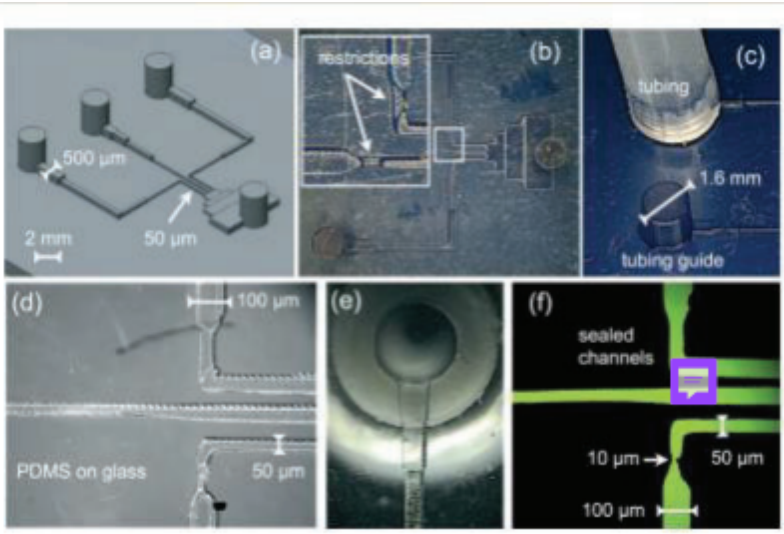

Figure 19. The 3D printing of chip template. (Filippini, 2019)

With the rapid development of 3D printing technology, a great deal of materials are being developed and selected, including translucent or transparent materials such as PDMS, PLA (polylactic acid) and transparent resins. Among them, PLA material is mostly used. PLA has the advantages of translucent color and glossy texture, and it is the best raw material for 3D printing chips at present. The microfluidic chip directly constructed by 3D printing is suitable for culturing a variety of cell and tissue types, simulating different in vivo microenvironments, and conducting in vitro physiological or pathological mechanisms and drug action mechanisms. Lu (2014) have designed a double-layer microfluidic chip with cell culture tanks. Firstly, melted maltose is used as the printing material to print the microchannel structure of the chip. After the sugar filaments are deposited, the PDMS material and the bubble-free PDMS material and the mixed solution of the curing agent is poured on the deposited sugar filaments, the poured PDMS is placed in a vacuum drying box for heating and curing, the cured chips are directly placed in boiling water to remove the printed sugar filaments and the chips can be obtained by washing. By culturing human breast cancer cells on the printed chip, a 3D dynamic culture chip model of human breast cancer cells was established.

On the other hand, biocompatibility is a fundamental prerequisite for all materials introduced into the field of bioanalysis. Any potential negative effects must be

identified and ruled out, as these characteristics may cause irritation and/or allergic reactions. For the application of 3D printing materials, material properties that need to be considered include biocompatibility, gas permeability and the sterilization method that can be selected (Yi, 2017). In other words, 3D printed biomaterials such as polyethylene glycol diacrylate, polyethylene glycol dimethacrylate, alginate, methacrylate gelatin, hyaluronic acid, etc. have good biocompatibility and have been used to encapsulate neurons, muscle cells, fibroblasts and human mesenchymal stem cells by stereolithography (carvalho et al, 2021). In order for 3D printing equipment to be used in biomedical research and clinical environments, the resin materials used must be experimentally confirmed to be biocompatible.

Based on the above exposition and analysis of the literature review, including bacterial vaginosis, the development of organ on a chip, the technology of electrospinning and 3D printing, the author of this article have fully comprehended the necessary background and knowledge which is relevant to the project. Vagina on a chip as a burgeoning area in biomedical engineering, it mainly aims to solve the female reproductive health diseases, including the replacement and reduction of animal testing or animal derivatives and the testing of drug delivery.

2.7 Research progress of electrospun chitosan nanofiber membranes

Chitosan is a natural linear biological polysaccharide obtained by N-deacetylation of chitin. The amino group in it can easily obtain a free positively charged amino group, so as to chemically react with the negatively charged molecule and form electrostatic adsorption with the molecule. The N-deacetylation degree of chitosan is an important performance index of chitosan, which will determine its performance to a certain extent. The weak alkaline property makes chitosan insoluble in water, but soluble in acidic solutions (pH < 6.5). Acid aqueous solutions convert the sugars in chitosan into soluble amino groups, allowing chitosan to dissolve smoothly (cui et al, 2021). The biodegradability of chitosan makes it widely used in the fields of biology and drug development. Its non-toxicity and antibacterial properties enable it to provide a good physiological micro environment for cell growth, while its adsorption can effectively increase the feasibility of cell growth in vitro on the scaffold. In addition, chitosan has a wide range of sources, high yield, second only to cellulose

and low cost. Its high content of amino and hydroxyl groups shows strong adsorption capacity for heavy metals, dyes and other organic substances, which has attracted the attention of scholars.

2.7.1 Chitin Electrospinning

Chitosan is obtained by deacetylation of chitin, which has similar chemical structure to cellulose. chitin has limited solubility, but chitin can be dissolved in 1,1,1,3,3,3-hexafluoro-2-propanol (HFIP) to prepare nanofibers by electrospinning. On the other hand, in order to enhance the solubility of chitin in hexafluoroisopropanol, the degradation of chitin can be enhanced by V-ray irradiation to reduce its molecular weight (Augustine et al, 2020). For example, setting the voltage to 15 kv, the inner diameter of the syringe needle of the mixed solution of chitin is 0.495 mm, and the concentration range of the chitin solution is set to be 1 wt% to 6 wt%. Spinning was performed under these conditions, and the resulting fiber membrane was collected on a collector approximately 7 cm away. The obtained electrostatic nanofibers were observed by SEM, and the following conclusions were drawn: at 3 wt% solution concentration, larger irregular beads or bead-like fibers were obtained by electrospinning; only at 4 wt% solution concentration, only continuous nanofibers can be made, and this concentration appears to be strongly related to the apparent chain entanglement in the solution. At a solution concentration of 6 wt%, because the solution of chitin has a very high viscosity (5310 mPa.s), continuous and uniform electrospinning was produced, but the presence of smaller beads in the fiber structure was also observed. The prepared chitin-based nanofibers had an average diameter of 110 nm as measured by observation. On the other hand, Noh (2006) et al. also produced chitin-based (chitin-N) nanofibers by electrospinning in HFIP and applied them to biodegradable wound dressings or scaffolds for tissue engineering. The electrospinning conditions were as follows: the concentration of chitin solution was 3 wt%-6 wt%, the electrospinning voltage was set at 17 kv, and a syringe needle with an inner diameter of 0.55 mm was used. As a consequence, in the application of electrospinning fibers to biomedicine, its high degradation rate has become its great advantage. The researchers attribute this feature to the fiber's low molecular weight rather than its smaller diameter. The experimental results showed that chi-N began to degrade 14 days after implantation in the body, which also indirectly proved that chitin fiber

matrix can be effectively used in the development and regeneration of wound dressings.

2.7.2 Chitosan Electrospinning

Based on the advantages of electrospinning technology, nano-ization of chitosan powder can further develop and expand the application value of chitosan. Due to the structural characteristics of chitosan itself, there are a large number of hydrogen bonds in its molecules, resulting in poor solubility and spinnability. In the current related research, although pure chitosan nanofiber membranes have been reported by electrospinning technology, most of them use toxic volatile solvents to dissolve chitosan. For example, trifluoroacetic acid, trifluoroacetic acid/dichloromethane mixture and more than 90 % high concentration acetic acid solution, etc. These toxic solvents cause a great deal of pollution to the environment and are difficult to remove. In addition, due to the high crystallinity of chitosan and the effect of intermolecular hydrogen bonds, the bonding of chitosan in solution and the behavior of polycations seriously hindered the spinning process, and the repulsive effect between polycations prevented the fibers from forming the desired chain entanglement and produce beads, which in turn result in poor fiber structure and extremely low deposition rates.

In 2004, Kousaku Ohkawa first spun chitosan into nanofibers by electrospinning. He dissolved chitosan (DD=78) in trifluoroacetic acid to make a 7 wt% spinning solution, and made continuous fibers under the condition of a voltage of 15 kv. Since then, many researchers have begun to study chitosan electrospinning. Trifluoroacetic acid cannot only form a salt with the amino group of chitosan, but also effectively reduces the interaction between chitosan molecules and makes spinning easier. Moreover, the high volatility of trifluoroacetic acid is also conducive to the rapid solidification of the electrostatic jet of the chitosan-trifluoroacetic acid solution, so it can be used as a solvent for electrospinning chitosan. But at the optimal concentration of 8 wt% chitosan solution, small beads and interconnected fibers can still be seen. Adding dichloromethane (dichloromethane/trifluoroaceticacid=30/70) to the chitosan-trifluoroacetic acid solution can improve the uniformity of chitosan electrospinning.

In recent years of chitosan electrospinning research, the solvent used is basically trifluoroacetic acid or acetic acid, or a small number of other solvents are added based on the two solutions. For example, in order to improve the stability of the chitosan electrospun membrane, Biranje (2017) et al. used saturated sodium carbonate solution to neutralize the chitosan electrospun membrane prepared by trifluoroacetic acid as solvent, which improved its neutral or weak performance stability in alkaline solutions. Schauer (2007) et al. used glutaraldehyde cross-linking method to improve the stability of chitosan fiber membrane. They obtained glutaraldehyde-crosslinked chitosan electrospun membranes by cross-linking during spinning and post-spinning cross-linking. Furthermore, the chitosan electrospun fibers were cross-linked with different cross-linking agents, and the mechanical properties after cross-linking were tested. Desai (2008) et al. obtained nanofibers with diameters of 80 ± 35 nm by heating-assisted and blending with PEO using acetic acid as a solvent. They studied the morphology and surface properties of chitosan nanofibers in detail, and also studied the electrospinning process of blended chitosan and polyacrylamide under the condition of heating assistance which also obtained blended fibers with better morphology. On the other hand, Satyajeet S. Ojha (2008) prepared chitosan/PEO core-shell fibers by coaxial spinning, and then after dissolving the PEO in the outer shell, pure chitosan fibers with a diameter of about 100 nm were obtained. However, this method is more troublesome to operate, and it is difficult to enlarge the application.

Geng (2022) successfully spun uniform fibers with an average diameter of 130 nm at 90% acetic acid mass fraction without beading. However, concentrated acetic acid has strict requirements on chitosan raw materials: only when the molecular weight is about 1.06×10^5 g/mol, the degree of deacetylation is about 56 and the mass fraction is 7 wt%-7.5 wt%, bead-free nanofibers can be obtained. When the electric field strength is in the range of 3-4.5 kv/cm, it can be spun into uniform and regular fibers; when the electric field strength is too small (less than 1 kv/cm) or too large (greater than 4.5 kv/cm), the fibers are not uniform and continuous. Vrieze (2007) et al. also used formic acid, acetic acid, lactic acid and hydrochloric acid as solvents to conduct electrospinning research on chitosan, and the results showed that nanofibers could be spun only when concentrated acetic acid was used as solvent.

Horzum et al. (2016) used hexafluoroisopropanol as a solvent to electrospin chitosan (molecular weight Mv=1096 kDa, deacetylated 70-75). At 1.1kv/cm, 0.4 wt% chitosan was spun into continuous uniform fibers with an average diameter of 42 nm without any defects such as beads. However, the solvent trifluoroacetic acid is corrosive and hexafluoroisopropanol is expensive. Therefore, some researchers add some polymers that are easy to form silk into the chitosan solution. For example, Rijal (2017) et al. blended Polyoxyethylene (PEO) with chitosan to make Cs/PEO nanofibers with an average diameter of (62±9) nm to (129±16) nm. In addition, synthetic polymers that are mixed with chitosan to make electrospun nanofibers include Polyethylene terePhthalate (PET), cellulose, PolycaProlactone (PCL), nylon-6 (Polyamide-6), Polylactic acid (PLA), etc.

Chapter 3. Preparation of chitosan/polyvinyl alcohol bioscaffold

3. Preparation of chitosan/polyvinyl alcohol bioscaffold

This section focuses on the fabrication process of the electronspun membrane which can provide the environment and space to culture the cells. As mentioned above, the membrane needs to provide excellent biodegradability and biocompatibility which can benefit the culture of the cells. On the other hand, the membrane needs to provide mechanical property to simulate the microenvironment in vagina. Previous material used in experiments for the electrospinning solution was PCL/gelatin mixture and its purpose was to verify the experimental feasibility of previous researchers on this project. Based on the background, chitosan was chosen as it can provide excellent biodegradability in the constructed vagina on a chip platform. Besides, (poly(vinyl alcohol), PVA) is insoluble in water at room temperature and is easy to combine with chitosan through hydrogen bonds, and has better electrospinning performance than other polymers. Its blending can produce continuous and defect-free nanofibers with high specific surface area that are insoluble in water, which is tougher and more durable than the film prepared by pure chitosan, and the cost of raw materials is also lower. Consequently, the following experiment details investigate the effects of chitosan deacetylation degree, Cs/PVA ratio, solution concentration and solvent concentration on the spinning morphology, the Cs/PVA electrospinning process was optimized which the optimal conditions for electrospinning Cs/PVA nanofiber membranes were determined. Then, the properties of Cs/PVA under optimal conditions were characterized by means of sEM, XRD, and the properties of electrospinning Cs/PVA were determined.

3.1 The structure and properties of chitosan

Chitosan is a linear biomacromolecule polysaccharide with amino groups, obtained by deacetylation of chitin. Chitin is an important renewable resource in nature, widely found in the skeletons of insects, crustaceans and mollusks, the skeletons of some algae and the cell walls of some fungi. Chitin exists in large quantities, with a content of up to 15%-30% in shrimp and crab shells alone. It is the most abundant organic natural polymer material, second only to cellulose in terms of production.

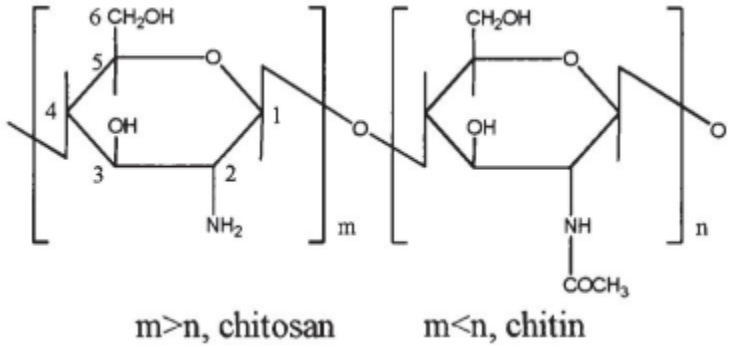

Figure 20. The structural formulas of chitin and chitosan. (Ohkawa, 2004)

Figure 20 shows the chemical formulas of chitosan and chitin, both of which are a type of polymer formed by connecting N-acetyl-D-glucosamine and D-aminoglucose through β-1,4 glycosidic bonds. When the content of D-aminoglucose structural unit (m) accounts for a large proportion in the molecular chain, it is called chitosan. On the contrary, when the content of N-acetyl-D-glucosamine structural unit (n) accounts for a large proportion, it is called chitin. The physical properties of chitosan mainly depend on the molecular weight, deacetylation degree (50%-95%), the arrangement structure of amino and acetylamino groups and the purity of the product, which can be characterized by solution viscosity, glass transition temperature and solubility. Generally speaking, the smaller the molecular weight of chitosan, the higher the degree of deacetylation, the less entanglement of its molecular chain, the lower the viscosity and the better the solubility. Due to the presence of a large number of intramolecular and intermolecular amino bonds in chitosan, its glass transition temperature is relatively high.

As shown in the chemical formula of Figure 20, the structure of chitosan is composed of glucosamine units, each of which contains an amine group (-NH_2) and a hydroxyl group (-OH), which can undergo esterification and etherification reactions with other compounds. These functional groups give chitosan specific chemical properties, which play an important role in applications in different fields. Chitosan has many advantages, including good biodegradability and biocompatibility and is environmentally friendly. It also exhibits good adsorption properties and can bind to and remove harmful substances. In addition, chitosan also has certain biological activities, such as antibacterial, antioxidant, and wound healing. However, chitosan also has the problem of poor solubility and low solubility in conventional solvents.

Researchers have modified chitosan, and common chemical modification methods include graft copolymerization, oxidation, and cross-linking reactions with multivalent cations and/or cationic polymers. These methods can change the chemical structure and properties of chitosan to meet the needs of specific applications. For example, graft copolymerization can introduce other functional groups to increase the solubility and reactivity of chitosan; oxidation can introduce aldehyde or hydroxyl groups to enhance the water solubility and biological activity of chitosan; and cross-linking with multivalent cations and/or cationic polymers can improve the mechanical strength and stability of chitosan.

The chitosan solution prepared in electrospinning exists in the form of a non-Newtonian fluid, which increases the difficulty in the electrospinning process. In addition, the non-Newtonian fluid with too high a viscosity affects the fiber forming degree of the needle jet during the electrospinning process, and it is difficult to form a Taylor cone to achieve a better spinning environment. The purpose of graft copolymerization is to introduce functional groups of other materials, thereby improving the solubility and reactivity of the chitosan solution. Therefore, polyvinyl alcohol, as a bio-friendly synthetic polymer material, can effectively improve the spinnability of chitosan.

3.2 The structure and properties of polyvinyl alcohol

Figure 21 shows the molecular structure of polyvinyl alcohol, which clearly shows that the PVA macromolecule has a planar zigzag structure similar to the traditional polymer material polyethylene. The main chain is all carbon atoms, the molecular structure is highly regular, and each PVA monomer contains a hydroxyl group, which leads to a large number of strong hydrogen bonds and strong polarity within and between molecules. The hydroxyl group is small in size and will not cause stress to the crystallization of the material. Therefore, PVA has a high degree of crystallinity and barrier properties, as well as excellent solvent resistance and oil resistance. It is soluble in water and polar solvents containing hydroxyl groups, and is miscible with polymers such as starch, plastic, and cellulose.

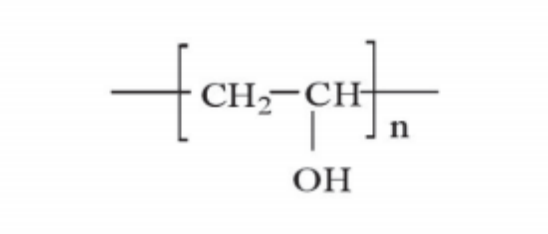

Figure 21. Polyvinyl alcohol molecular structure. (Wang, 2015)

As one of the commonly used polymers in the biomedical field, PVA is used as a carrier for drug delivery due to its non-toxic, non-carcinogenic, and good biocompatibility. On the other hand, the film made of PVA material has strong surface stability and a rich pore structure, which also increases its wide application in biomedicine. The molecular structure characteristics of high hydroxyl content (≥ 85%) allows PVA to have good water solubility. In the field of electrospinning, when it is combined with other polymer materials, it can greatly improve the viscosity of the polymer solution, thereby promoting the electrospinning process.

Based on the above description of the chitosan and polyvinyl alcohol structural units, it clearly shows the characteristics of the two different polymer materials. This also provides a theoretical basis for the electrospinning fibers combining two different polymer materials in this project. Biodegradability and biocompatibility are the prerequisites for the application of the two materials in biological scaffolds. They can ensure that the scaffolds made of the two materials are harmless to the human body and suitable for human application. Secondly, the good mechanical properties provided by the amino and hydroxyl functional groups ensure that the biological scaffolds are suitable for the growth of vaginal epithelial cells, thereby simulating the physiological microenvironment of the peristalsis of muscle tissue inside the vagina. Thirdly, the addition of polyvinyl alcohol triggers the chemical reaction of the functional groups inside chitosan, thereby effectively improving the solubility of chitosan in the electrospinning process, so as to better prepare nanofiber scaffolds. In summary, chitosan and polyvinyl alcohol are used as the main materials used in this project to prepare scaffolds to provide a three-dimensional growth site for in vitro culture of vaginal epithelial cells and simulate the physiological microenvironment that can be provided inside the vagina.

3.3 Electrospinning Experimental section

3.3.1 Experimental Materials

Tables 1 and 2 show the experimental materials and related instruments used in the electrospinning part of this project, including the molecular weight, specifications and manufacturers of the materials.

Reagent name	Specification	Molecular weight	Manufacturer
Chitosan	Deacetylation 75-85%	50,000-190,000 Da	Sigma-Aldrich
Polyvinyl alcohol	A.R	80000	Sigma-Aldrich
Acetic acid	A.R	60.05	Sigma-Aldrich

Table 1. Experimental Chemicals and Medicines Form

Name	Manufacturer
Electronic Analytical Balance	METTLER TOLEDO
Magnetic stirrer	Carl Roth
X-ray diffraction analyzer (XRD)	Bruker
Vacuum drying oven	Drawell
Extensometer (Modular Force Stage with a 20N load cell)	LinkAM UK
Fourier-transform infrared spectroscopy(FTIR)	Thermo Fisher scientific
Scanning electron microscopy (SEM)	ZEISS

Table 2. Experimental Instrument Usage Form

3.3.2 Preparation of electrospinning solution

3.3.2.1Method for preparing spinning solution

In this article, chitosan and polyvinyl alcohol composite nanofibers were prepared by electrospinning technology. The basic preparation process was as follows: A certain amount of chitosan was weighed and dissolved in an aqueous acetic acid solution, and

stirred uniformly by magnetic force to prepare a chitosan solution. 10 wt% polyvinyl alcohol aqueous solution was dissolved in a water bath at 80°C, blend in different proportions, disperse uniformly by ultrasonic, and let stand for defoaming to obtain spinning dope. The purpose of adding the solution of polyvinyl alcohol was in order to improve the spin ability of the chitosan solution, so that the spinning solution can better burst into filaments from the needle. On the other hand, it increases the hydrophilicity of the polymer to a certain extent and improves the chemical properties of the polymer. It is worth noting that in the study of this project, the concentration of polyvinyl alcohol was constant, which did not affect other parameters of the spinning process and the fibrillation results of the electrospun membrane.

Firstly, an acetic acid solution was prepared, then a prescribed amount of chitosan powder was weighed into the acetic acid solution, which was placed in a magnetic stirrer for stirring. Setting the rotating speed of the magnetic stirrer was 500 rpm and the stirring temperature to 30 degrees. Rotating chitosan in the beaker for 10 to 12 hours and observing the condition of the spinning solution, if the solute was not completely dissolved and continue stirring until the solute was completely dissolved. Secondly, dissolving polyvinyl alcohol powder in ultra-clean water in a water bath heated to 80°C and placing it on a magnetic stirrer to stir for 2-3 hours to observe the color and state of the polyvinyl alcohol solution. It was stirred until it is transparent without other powders, which means the dissolution is complete. Chitosan solution and polyvinyl alcohol solution were mixed according to the volume ratio of 2:8 and placed on a magnetic stirrer to stir for 2 hours, then left to stand until the solution was completely fused before being used for electrospinning.

In order to explore the concentration of chitosan solution suitable for the growth of vaginal epithelial cells, this project was divided it into four groups of different concentrations, namely 2 wt%, 3 wt%, 4 wt% and 5 wt%. Four different concentrations of chitosan solutions were added with a constant 10 wt % polyvinyl

alcohol solution to improve the solubility and spinnability of the chitosan solution, so as to explore the most suitable spinnable solution parameters.

3.3.2.2 Preparation method of high concentration chitosan solution

It should be noted that in the process of preparing the solution, as the concentration of the chitosan solution increases, its viscosity increased accordingly, making the solubility of chitosan in the acetic acid solution less. For example, when the solution concentration increases to 5 wt%, it was clearly seen that the chitosan is dispersed into white precipitates of varying sizes in the acetic acid solution. In order to solve this phenomenon and ensure the smooth dissolution of chitosan, the author explored different methods to ensure that the chitosan solution is smoothly stirred into a spinning solution. Firstly, a method of mixing using high and low speeds was adopted. When a high concentration of chitosan was just added to the acetic acid solution, the speed of the electronic stirrer was adjusted to 300rpm. The purpose of this operation was to quickly disperse the chitosan powder to prevent it from forming a large amount of white precipitate in the acetic acid solution. The chitosan powder was dissolved at a high speed for thirty minutes, and the chitosan solution gradually turned into a non-Newtonian fluid. At this time, the state of the blending of the chitosan powder was observed with the acetic acid solution, and the speed of the stirrer was gradually reduced to 100rpm. The purpose of the low speed is to allow the rotor in the stirrer to rotate smoothly in the non-Newtonian fluid, thereby helping to dissolve the white precipitate in the solution. Stir at a low speed for 12 hours, and observe the blending state of the solution until the chitosan powder is completely dissolved.

The second method was to add chitosan powder in batches. According to the solution ratio calculation formula, 5wt% chitosan solution needs to add 0.52 grams of chitosan powder in 10ml of acetic acid solution. It can be added in the form of portion, for example, 0.2 grams of chitosan powder was weighed and added to the acetic acid solution, and then 0.2 grams of chitosan powder was added after it is completely dissolved, and the last 0.12 grams of chitosan powder was added until it was completely dissolved. This method was the most effective operation for dissolving high-concentration chitosan powder. The method of adding in batches can effectively reduce the white precipitate in the blended solution, avoid the solution

from forming a non-Newtonian fluid too early, thereby increasing the feasibility of stirring.

3.3.3 Electrospinning process

3.3.3.1 Electrospinning membrane preparation process

After the spinning solution was prepared, the next step was implementing electrospinning. In the process of electrospinning, it was divided into two parts the solution propelling device and electrospinning equipment. The solution was drawn into the syringe through the needle slowly. The solution was sucked by the syringe through the needle, then the syringe pump was slowly pushed manually until the spinning solution slowly flows out from the needle and the spinning solution at the needle tube is sucked up with suction paper. Then, putting the syringe which filled with the spinning solution on the propelling device and adjusted the distance between the syringe needle and the delivery device.

After the propelling device starts, enter the main interface of the machine control parameters to set the relevant parameters, including the propulsion speed of syringe pump and the propulsion time. The propulsion rate was 0.2 mL/hour and propulsion time was 6 hours. Figure 22 exhibits the electrospinning process. The infusion channel was connected with the electrospinning launching device and the syringe pump which guarantees progress of the infusion process. Then the front end of the infusion channel installs the needle which is held by the clamp of the launching device, the needle and clamp is generally placed at 90 degrees. At the bottom of the electrospinning machine is the collector device which behave as the silver disc. Before starting the electrospinning, the silver disc needs to be covered with a layer of tin foil which can collect the superfine fibres spun by the high voltage to form fibre mat. On the other hand, in order to prevent the spinning solution from spinning smoothly from the launching device, it is necessary to place a beaker with water under the needle before electrospinning. Then driving the injection pump slowly until the spinning solution flows out of the needle into the beaker to guarantee the spinning solution can flow smoothly in the infusion channel.

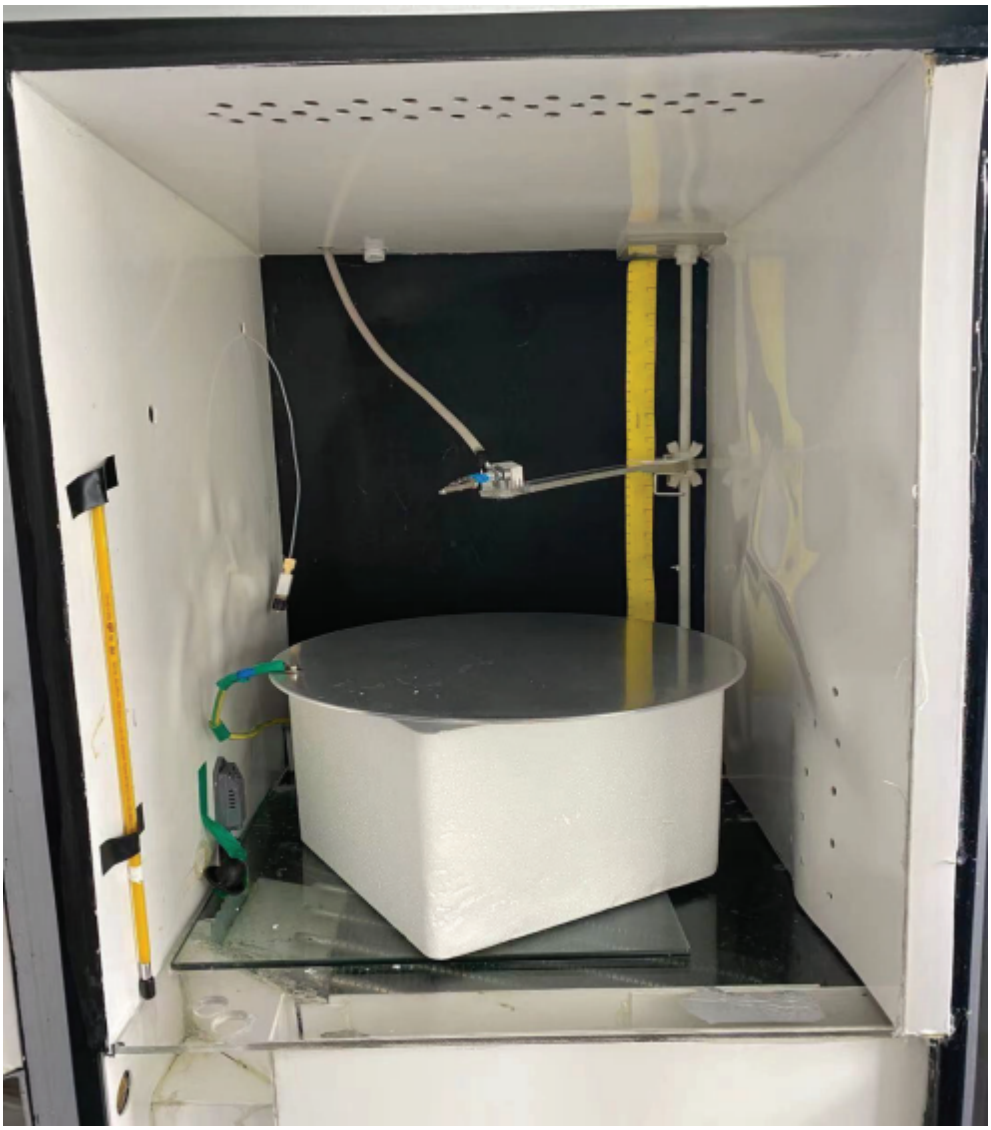

Figure 22. The electrospinning equipment in laboratory

With the preparing work, adjusting the voltage parameter to 20-25 KV and closing the door of the electrospinning machine. Then the spinning solution can spin uniform on the collector and form to the fibre mat. During the electrospinning process, the needle may be entangled with fibres which can cause uneven fibers to be spun from the needle and affect the characterization of the fiber membrane. When the needle was entangled with fibers, the power needs to be turned off, open the glass door and clean the needle to guarantee the normal progress of the spinning process. Therefore, it was necessary to regularly observe the spinning state of the needle through the glass door. The electrospinning process lasts for 6 hours generally. Figure 23 shows the membrane which spins by the machine.

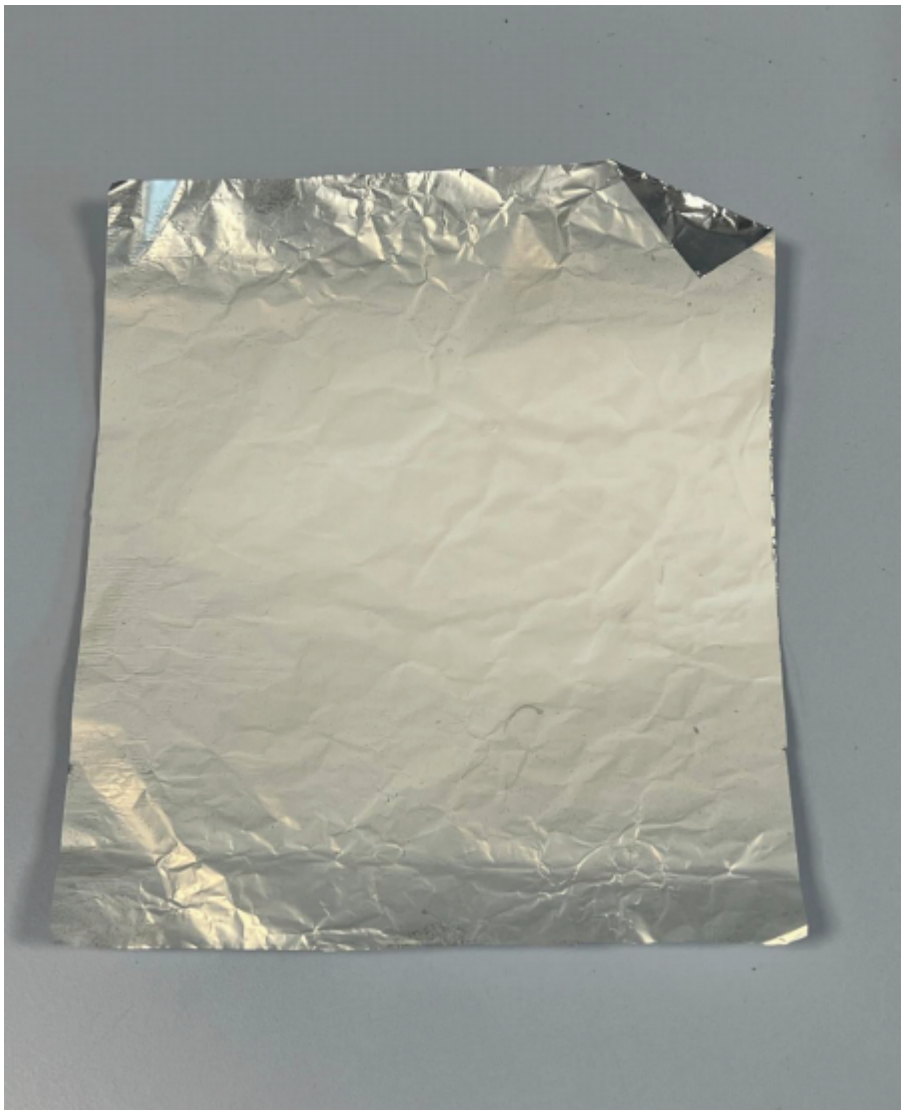

Figure 23. The chitosan electrospinning membrane

3.3.3.2 Precautions for electrospinning process

As mentioned above, the characteristics of chitosan materials are that its solution is a Non-Newtonian fluid with low solubility, which means that this material is much more difficult to electrospin than other materials. In other words, the difficulty of electrospinning means that the control of parameters during the spinning process must be more stringent, including parameters such as voltage, humidity, solution flow rate and receiving distance. Therefore, this section will summarize the problems that occur during the chitosan spinning process and explain the corresponding solutions for reference.

The first issue that needs to be mentioned was the adjustment of the solution flow

rate during the spinning process. Limited by the characteristics of the chitosan solution, although the addition of polyVinyl alcohol solution improves its solubility and chemical reactivity, its solution flow rate is not constant. In the early stage of spinning, under the action of high voltage, the solution quickly gathered at the needle and sprayed out irregular nanofibers, but no regular Taylor cone shape was formed. At this time, the solution flow rate is 0.2ml/h as mentioned above. After the fibers are sprayed out, the flow rate should be adjusted to 0.1ml/h, or even lower. The purpose of adjusting the solution flow rate is to prevent the droplets from being sprayed out too fast under high voltage and affecting the morphology of the nanofiber membrane. On the other hand, the chitosan solution fluid pressure at a low flow rate is smaller, which can help the non-Newtonian fluidflow more smoothly, thereby ensuring a smooth spinning process of the chitosan solution.

The second problem was the control of spinning voltage. Electrospinning voltage that was too high or too low will affect the morphology of the nanofiber membrane during the spinning process. Low voltage made it difficult for the electric field force to overcome the surface tension of the solution, thereby affecting the stretching effect of the solution under the action of voltage, making the electrospun fibers discontinuous. Excessive voltage increased the stretching force of the electric field force on the solution, bursting out tiny discontinuous droplets, and thus forming beaded fibers. Therefore, in the spinning process of chitosan, the voltage needs to be increased (greater than 20kv) at the beginning of spinning to enable the electric field force to continuously stretch the solution and form continuous and uniform fibers; after the fiber output is stable, the voltage is gradually reduced (but not less than 18kv) to ensure that no droplets appear.

Humidity during the spinning process is a factor that is difficult to control, and the adhesion or fiber hardening at the spinning needle is related to humidity. Too low humidity will increase the volatility of the solution. The solution that evaporates too quickly would deposit at the needle to form sticky white fibers. These sticky white

fiber substances will block the needle to a certain extent, thus affecting the normal fiber production. Excessive humidity will slow down the evaporation of the solution. Under the high voltage stretching effect, the solution formed tiny droplets that gathered on the needle surface. These aggregated droplets were sprayed onto the surface of the nanofiber membrane under the action of the electric field force, forming beaded fibers or droplet deposition, which seriously affects the fiber morphology. Therefore, in the electrospinning process of chitosan, in order to effectively control the change of humidity in the spinning chamber, 250 ml of pure water was placed in the spinning chamber, and the humidity change was observed through the humidity control system of the electrospinning machine. For example, when the humidity is too high, the pure water will be taken out to dry the air in the spinning chamber. This also means that the spinning process is a complex and changeable process, and the spinning environment needs to be adjusted in time according to the changes in relevant parameters to ensure the smooth spinning and good fiber morphology. Based on the complex spinning environment and the characterization performance of electrospun fibers, the best result was achieved when the spinning humidity is controlled between 50% and 56%.

3.4 Crosslinking

Due to the hydrophilic characteristics of polyvinyl alcohol, the spun nanofibers dissolve quickly in the liquid which will affect the subsequent cell culture work. Therefore, the purpose of crosslinking was to modify the spun nanofibers so that they can exist in liquids. Glutaraldehyde is active in nature which is easy to polymerize and oxidize and react with compounds containing active oxygen and compounds containing nitrogen. The reaction with protein is mainly the reaction between carbonyl group and amino group in any protein molecule. Among the known aldehydes, glutaraldehyde is an excellent protein cross-linking agent. Glutaraldehyde has excellent bactericidal properties. Glutaraldehyde has little effect on the activity of this enzyme, and most enzymes can be immobilized and cross-linked without inactivation under controlled conditions.

The cross-linking of chitosan and polyvinyl alcohol nanofibers was carried out in a fume hood. The specific operation process is to pour 10-15 ml of glutaraldehyde solution into the petri dish, and place the petri dish at the bottom of the desiccator. Fixing the

electrospun film to the top of the petri dish, pumping the desiccator to a vacuum state, and then placing it in a fume hood for 48 hours to complete the cross-linking. The cross-linking treatment of chitosan/polyvinyl alcohol electrospun membrane by glutaraldehyde enables the nanofibers to exist in liquid, so that they cannot be dissolved. This provides support for handling electrospun membranes, including membrane stripping work and cell culture work.

3.5 Oriented electrospinning

Based on the above exploration of chitosan/polyvinyl alcohol electrospinning parameters and the explanation of problems encountered during the spinning process, the project continued to explore whether oriented electrospinning has a promoting effect on the growth of vaginal epithelial cells under the above conclusions. Within the project the differences between oriented scaffolds and non-oriented scaffolds explored. Oriented electrospinning is a spinning method based on traditional electrospinning, which changes the orientation of fibers under the influence of external equipment or conditions. Through guidance under certain conditions, the fibers on the collector are changed from a disordered state to an ordered state, and have an orientation in a certain direction, so that the fibers have better performance characteristics. The oriented electrospinning work of this project was completed in 2023 while at Zhongyuan University of Technology.

The chitosan/polyvinyl alcohol spinning solution at the optimal concentration of 4 wt% was selected for oriented electrospinning, and oriented electrospinning was attempted under the same voltage, receiving distance and humidity parameters. The main method was to replace the original static receiving device with a roller at a fixed speed of 500 rpm, which rotated at a constant speed to receive the fibers ejected by the needle.

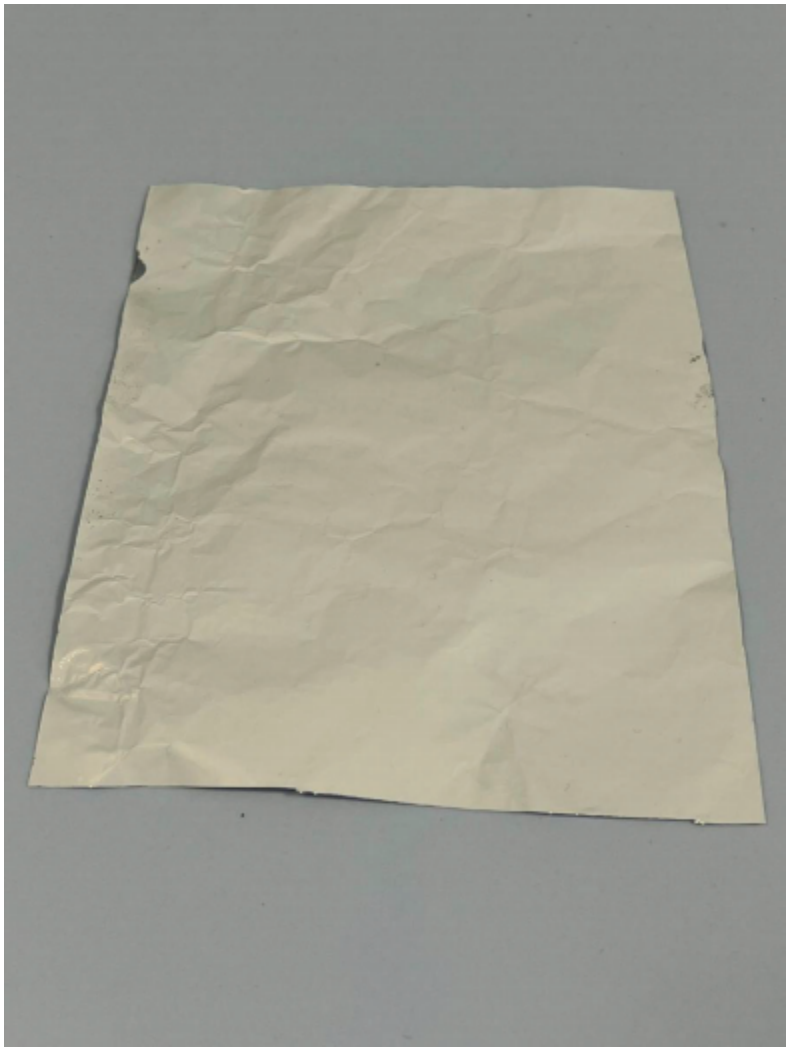

Figure 24. oriented electrospun chitosan/polyvinyl alcohol membrane

Figure 24 shows the collected oriented electrospun chitosan/polyvinyl alcohol film, which clearly shows that its surface morphology is complete and smoother. compared with the disordered chitosan/polyvinyl alcohol film, the oriented film shows better texture and morphological characteristics.

3.6 Evaluation of electrospun membrane properties

3.6.1 Scanning electron microscope to evaluate fiber morphology

The main working mode of the scanning electron microscope (SEM) is that when the incident electron hits the sample surface, different signals will be generated, including secondary electrons and backscattered electrons. These electron beams are focused by the electromagnetic lens and controlled by the scanning coil to scan the surface of the sample. The main scanning depth of the secondary electron beam is 5-10nm of the sample, and the backscattered electron beam is more than 100nm. The scanning depths of these different electron beams represent the different structural characteristics of the fibers

inside the sample. For example, the difference in sample surface morphology can be expressed by different contrasts, which are presented as brightness differences in different parts of the SEM image.

For these experiments samples were cut into 1.5cm x 1.5cm squares, these electrospun membrane were fixed on the stage, a conductive glue was used to adhere to the four corners to ensure that the electrospun membrane is accurately fixed. Then it is placed in the SEM for adjustment, including position adjustment and pattern capture.

3.6.2 Fibre Diameter Measurement

The SEM pictures (Figure 25) of electrospun membranes can show the characteristic morphology of fibers, including the distribution, diameter and size of fibers. Based on the results of the electron microscope pictures, the diameter data of 100 fibers were evaluated by ImageJ software, and five electron microscope pictures were taken on average for a single sample. Based on the fiber diameter data, the distribution curve of fiber diameter was obtained by using origin. Figure 25 shows the SEM diagram of the fiber measured by ImageJ. A total of 50 fibers were selected from the surrounding and middle areas of the SEM image, and the diameters were selected, and the data were exported for the next step of Origin analysis.

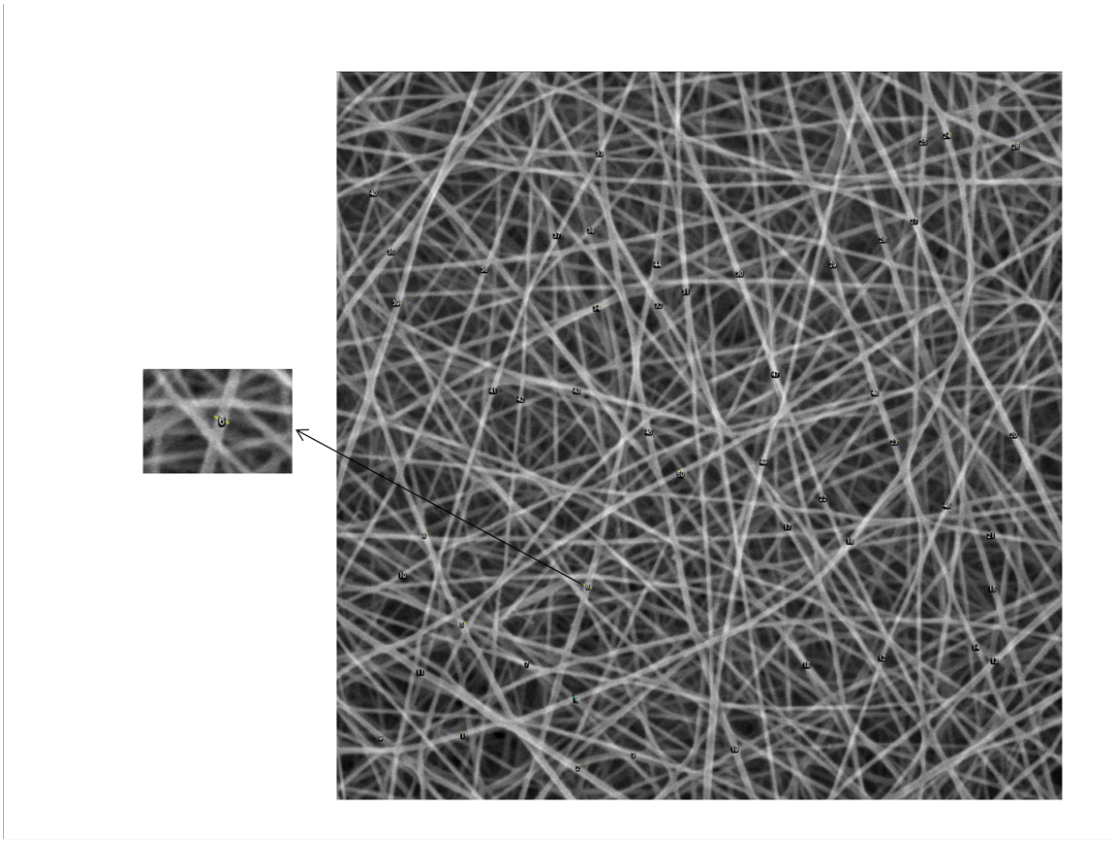

Figure 25. SEM fiber diameter measured by ImageJ

3.6.3 Stress-Strain Analysis

Under the action of external force and a non-uniform temperature field and other factors, the material can produce relative deformation locally. In the process of bearing additional pressure and strain, a force can be generated inside the material to counteract the external force and overcome the deformation. This internal force is stress. In other words, the force that a certain material bears per unit area is stress, which can also be called pressure. Strain-stress curves are often used to test a materialIs ability to resist deformation. In this test, the mechanical properties of the chitosan/polyvinyl alcohol electrospun membrane were tested and analyzed using a universal mechanical testing machine combined with LINK software, and its feasibility in simulating the internal mechanical characteristics of the vagina was explored. The maximum force of this machine is 20N. Table 3 shows the test parameter values of four scaffolds with different concentrations during strain stress testing.

Strain stress test parameter					
Name	Rate	Sample length	Sample width	Sample thickness	Average force(N)
CS2/PVA10	10.00 mm/min	50.0 mm	10.0 mm	8 μm	0.471
CS3/PVA10	10.00 mm/min	50.0 mm	10.0 mm	8 μm	0.823
CS4/PVA10	10.00 mm/min	50.0 mm	10.0 mm	8 μm	1.141
CS5/PVA10	10.00 mm/min	50.0 mm	10.0 mm	8 μm	0.508

Table 3. Strain-stress parameters of four scaffolds with different chitosan concentrations

The strain stress operation is to cut the electrospun membranes of four different concentrations into rectangular films with a width of 1 cm and a length of 5 cm, and use a thickness tester to measure the thickness of each film. It is worth noting that in the test of the sample, the total length of the sample is 5 cm, and the sample is clamped 1 cm at both ends, so the 3 cm position in the middle of the sample is the actual area of the strain stress test. The rectangular film is clamped and fixed on the strain stress tester to keep the film in a relaxed state so that it can be subjected to stress testing. The stress test stage lasts until the film is completely broken. Each concentration of film is tested five times, and the average result is taken for analysis.

3.6.4 Fourier transform infrared spectroscopy

Fourier transform infrared spectroscopy (FTIR) uses the property that different substances absorb light of different wavelengths to different degrees. It can effectively judge the composition of mixed materials or substances based on the absorption degree of the spectrum, thereby identifying each component of the blend. For organic materials, the essence of its measurement is the absorption degree of functional groups of different substances. For example, different functional groups have different corresponding absorption peaks, so the composition of the blend polymers

can be effectively judged based on the different components in materials functional groups.

To undertake infrared spectroscopy the chitosan/polyvinyl alcohol electrospun membranes of four different concentrations were dried in a vacuum drying oven for two hours, cut them into 1cm x 1cm squares, and then carefully peel each film from the tin foil with sterilized tweezers. The peeled electrospun membranes are placed in a special container for infrared spectroscopy and crushed, and then spectral powder is added to press them into flake crystals, which are placed in the machine for scanning to obtain infrared spectra. In the process of FTIR testing, the results are often presented in the form of spectra. Specific chemical bonds and functional groups of molecules in different samples absorb infrared radiation and cause molecular vibrations. Fourier transform technology converts these light signals into spectra to generate infrared spectra. In terms of data presentation, different absorption peaks at specific wavelengths represent different molecular chemical bonds.

3.6.5 X-ray diffraction analysis

The purpose of X-ray diffraction is to characterize the changes in the crystal structure of materials or substances. Its working principle lies in the interference of X-rays in the crystal. The energy of the X-rays themselves will cause the corresponding atoms in the crystal to make periodic movements, and this movement causes the atomic ball to emit secondary waves outward. The frequency of the incident X-rays is consistent with the frequency of the infrasound wave, thereby completing the scattering process of the X-rays. From the perspective of the crystal of the material, the spatial arrangement of the atoms in the crystal is regular. The scattering of X-rays and infrasound waves has a fixed phase relationship with the atoms arranged in the crystal, thereby causing interference in the space of the crystal, further strengthening certain scattered waves. These scattered waves will also produce a cancellation phenomenon in a certain direction, thereby realizing diffraction in the crystal. In short, its principle is the result of X-rays diffracting on the atomic surface of the crystal and interfering with each other.

X-ray diffraction has no fixed requirements for the size of the object, and the electrospun membrane is often cut according to the size of the X-ray diffractometer

fixing table to achieve the purpose of fixing. After fixing the electrospun membrane of each concentration, it is scanned at a selected wavelength to obtain the results.

3.7 Result Analysis

Chitosan composite nanofibers prepared by electrospinning technology have different spinning effects due to different process conditions, properties of spinning dope, and concentrations. This section mainly discusses the effects of process parameters such as electrostatic voltage, receiving distance, spinning solution flow rate, etc. On the fiber formation and morphology of chitosan composite nanofibers. fiber effects. The morphology observation and structure of chitosan composite nanofibers were analyzed by scanning electron microscope and X-ray diffraction, which provided a detailed reference for the preparation of chitosan composite nanofibers and aided a solid foundation for its further application.

3.7.1 Electron microscopic morphology of four different concentrations of scaffolds

The morphologies of the four chitosan/polyvinyl alcohol scaffolds with different concentrations reflect the degree of fiber entanglement and diameter thickness at different concentrations. The side also reflects that the concentration has different effects on the morphology of the scaffold. Figure 26 shows the morphological structure of four scaffolds with different chitosan concentrations (2, 3, 4 and 5wt%) under a scanning electron microscope. The morphological structure clearly shows that the concentration has different effects on the scaffold in Figure 26. (a) The chitosan morphology at a concentration of 2wt% is poor, and its surface is mostly disordered fibers, and beaded fibers are generated; (b) The fibers at a concentration of 3wt% begin to show a more regular morphology, and the beaded fibers are significantly reduced; (c) The morphology of the fibers at a concentration of 4wt% was the best, and the fibers have a certain degree of order, and the diameters of different fibers are relatively uniform and there are no beaded fibers; (d) The fiber morphology at a concentration of 5wt% also shows a more regular shape, without obvious beaded fibers, but the diameters of different fibers are uneven.

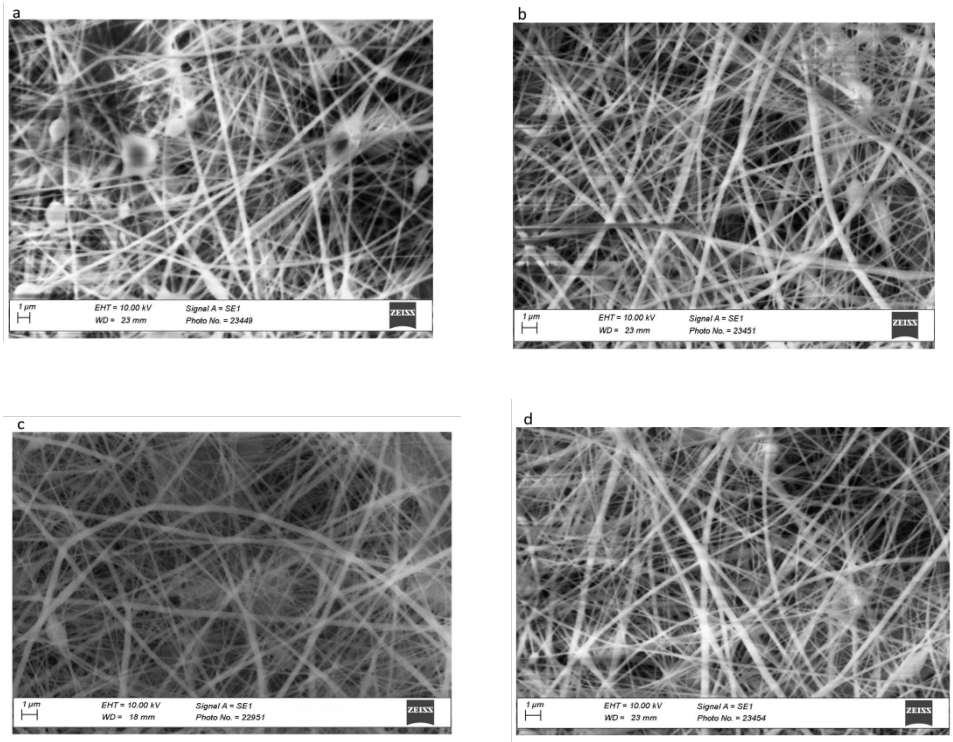

Figure 26. SEM images of chitosan/polyvinyl alcohol at four different concentrations. (a) CS/PVA 2%; (b) CS/PVA 3%; (c) CS/PVA 4%; (d) CS/PVA 5%.

From the above description of fibers with different chitosan concentrations, it can be seen that the fiber morphology at a concentration of 4wt% is the most regular, and its fiber diameter is more evenly distributed, the arrangement of fibers also shows more regularity. Secondly, the depth of field and imaging effect of the SEM picture can also indirectly illustrate the good degree of the fibers in the scaffold. The imaging effect of fibers with regular morphology and uniform arrangement is clearer and easier to image. On the other hand, the reason why the morphology of chitosan fibers is poor at low concentrations (2wt%-3wt%) is that the concentration of chitosan is low and the degree of reaction between it and the functional groups of polyvinyl alcohol is low, resulting in weak bonding between its crystalls, resulting in too many beaded fibers and uneven fibers diameters. When the chitosan concentration is too high (5wt%), the solubility of chitosan is reduced, and the macromolecular functional groups in the composite material are increased, which will hinders the reaction between the functional groups to a certain extent, making the fibers uneven and the diameter larger accordingly.

In addition, the author also investigated the fiber morphology effect of oriented electrospinning for the improvement of chitosan/polyvinyl alcohol fibers. Figure 27

shows the morphology comparison of oriented fibers and non-oriented fibers, both of which were tested at the optimal concentration of 4wt%. It can be seen that the arrangement of oriented fibers is more regular, the fibers are arranged in a certain orientation, and the fiber diameter is also thinner.

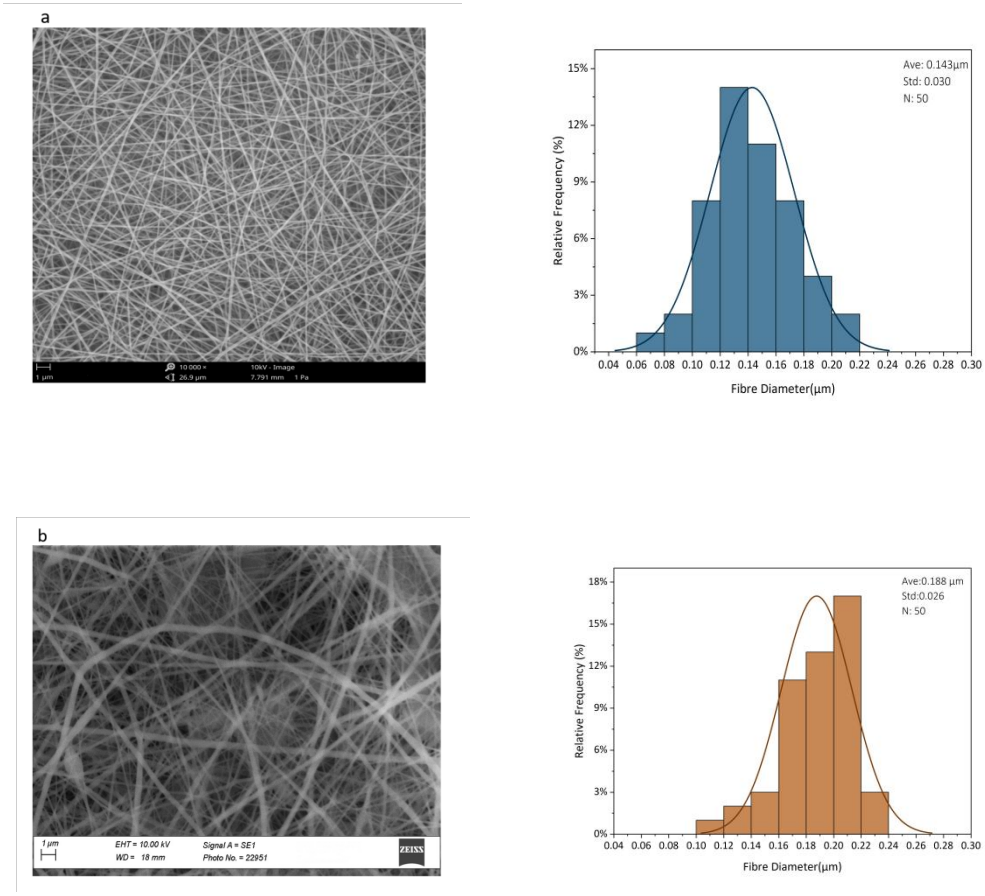

Figure 27. Comparison of SEM images and diameter of oriented and non-oriented fibers. (a)CS/PVA 4% oriented. (b) CS/PVA 4%

The fiber diameter distribution diagram on the right also clearly illustrates the distribution range of fibers in two different scaffolds. The fiber diameter of the oriented scaffold is mainly distributed between 100 nanometers and 160 nanometers, accounting for 80% of the fibers. The fiber diameter of the non-oriented scaffold is distributed between 160 nanometers and 200 nanometers, accounting for more than 90%. This data proves that the fibers of oriented electrospinning have a finer fiber diameter and a more uniform fiber arrangement. On the other hand, a finer fiber diameter means more fiber roots in the same area, which can provide better mechanical properties for the scaffold.

3.7.2 Optimal parameters of chitosan

According to the SEM images of chitosan in chapter 3.7.1 the optimal parameters for the preparation of chitosan nanofibers by electrospinning are as follows: 90 wt % acetic acid

was used as solvent, the concentration of chitosan solution was 4 wt %, the spinning voltage was 23 KV and the receiving distance was 6.8 cm. The obtained chitosan nanofibers had an average diameter of 180±25 nm.

As a consequence, chitosan is a fiber material that is biodegradable and has good cell compatibility. The fiber film made by electrospinning can be effectively used as a carrier for cell culture, so that cells can grow and differentiate normally. Chitosan is insoluble in dilute phosphoric acid and sulfuric acid, but soluble in dilute organic acids and some inorganic acids. It can also be dissolved in concentrated phosphoric acid, nitric acid and hydrochloric acid by heating and stirring for a long time, but is insoluble in water and alkaline solutions. The dissolved chitosan solution will be slowly hydrolyzed and the viscosity will decrease. The greater the acetylation of chitosan, the smaller the molecular weight and the greater the solubility. On the other hand, chitosan solution exhibits different viscosity characteristics due to different preparation conditions, which are mainly related to factors such as temperature, solvent type, concentration, pH value and other factors during preparation. The viscosity of chitosan solution mainly depends on the relative molecular mass, the greater the relative molecular mass, the greater the viscosity. The degree of deacetylation also has an important influence on the viscosity. cChitosan with a low degree of deacetylation is difficult to dissolve, and the viscosity also increases. Therefore, although the application of chitosan to electrospinning is feasible, the process is relatively difficult. The chitosan electrospinning film that can be applied to cell culture studied in this topic is also under continuous exploration. It will be improved by continuous optimization methods, such as cross-linking with other polymer materials to modify the properties of chitosan and improve its spinnability.

3.7.3 Comparative analysis of strain stress curve

In the previous study of this subject, the electrospinning film made by related researchers using PCL/gelatin mixed material has been proved to be used in the culture of vaginal cells, whereas the culture result is limited by the mechanical properties of the film. Therefore, in the process of continuing the subject research, chitosan is used to replace the previous material for cell culture. In this part, strain stress tests were performed on four different concentrations of chitosan/polyvinyl

alcohol scaffolds under optimal environments to explore the most suitable chitosan concentration for cell growth.

Figure 28 shows the strain stress curves of four different chitosan concentrations. It can be clearly seen that in the elastic stage, as the chitosan concentration increases from 2 wt% to 4 wt%, the maximum stress at which the material maintains complete elastic deformation also increases. When the concentration of chitosan reaches 5wt%, the maximum stress suddenly drops. This is because the increase in concentration increases the molecular weight in the material, resulting in greater intermolecular forces, resulting in a reduction in stress. In the yield stage, the linear relationship between strain and stress is destroyed. The stage where the curve strain increases significantly in the figure is the yield stage, which shows that the material no longer maintains elastic deformation in this part and begins to produce plastic deformation. It can be clearly seen that chitosan with increasing concentration bears greater stress until it breaks during the yield stage, but the stress at a concentration of 4wt% is the largest, which represents the maximum stress that the material can withstand before it breaks. In summary, the mechanical properties of chitosan are optimal when the concentration is 4%.

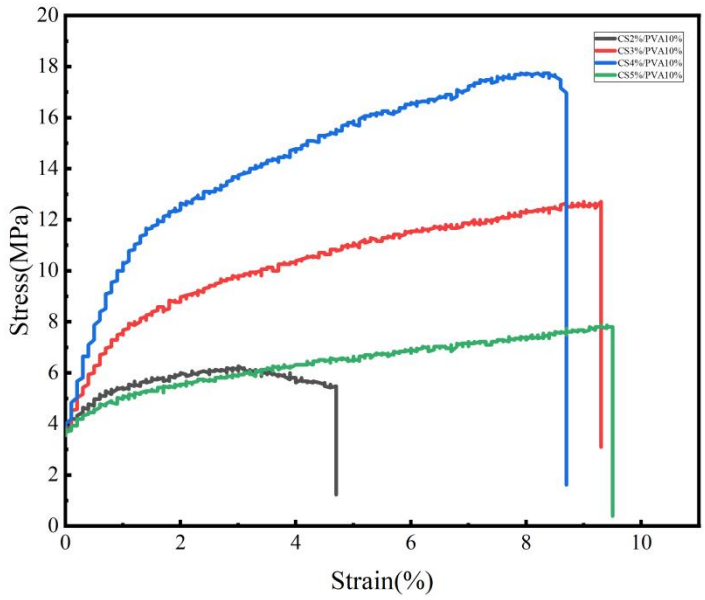

Figure 28. strain-stress curves of different concentrations chitosan/PVA electrospun membrane.

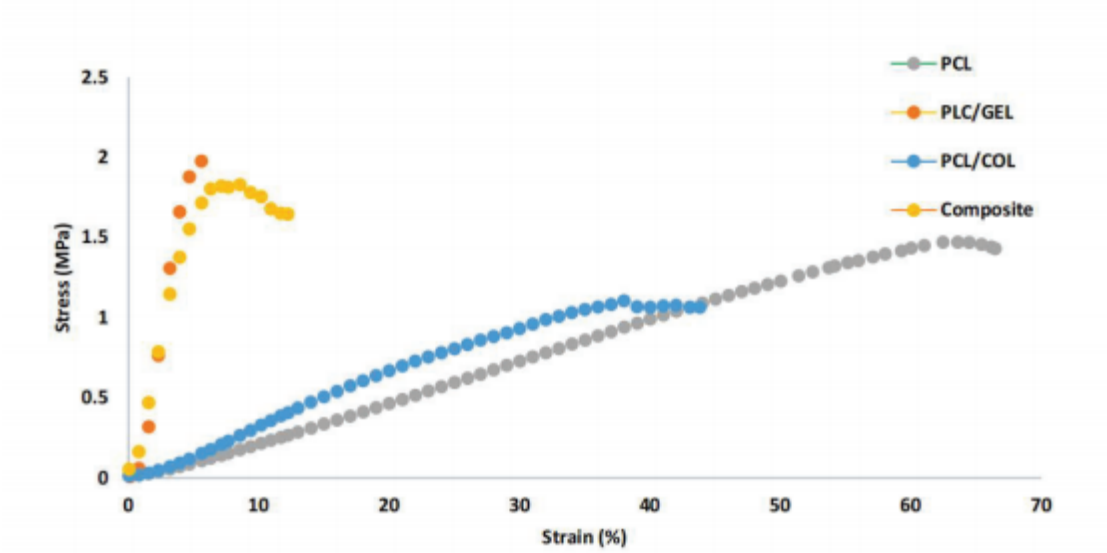

Figure 29. PCL/Gelatin strain stress test results (Naveenathayalan, 2021)

In this section, we have added the comparison of strain-stress results of scaffolds for vaginal epithelial cell growth by previous scholars. Naveenathayalan, 2021 used PCL/Gelatin electrospun membrane to grow vaginal epithelial cells. Figure 29 shows the strain-stress results of the electrospun membrane spun under ideal solution conditions. It should be noted that the comparison of strain-stress of the two electrospun membranes here is the result of data processing, that is, the calculated true stress and true strain data. Usually goes through four different stages, including the elastic stage, the strain stage, the strain hardening stage, and the fracture stage. The Young's modulus of the material is calculated based on the degree of change in the elastic stage of the material. Yield stage corresponds to the stress corresponding to the plastic deformation of the material. The yield stage curves of different materials vary greatly, and it is difficult to make a more accurate analysis. Generally speaking, for polymer materials, the Young's modulus in the elastic stage determines the mechanical properties of the material itself, which is represented by the ratio of stress to strain. The tensile strength represents the maximum tension or shear force that the material itself can withstand per unit surface. Figure 30 shows the Young's modulus values, tensile strength and elongation at break of four chitosan/polyvinyl alcohol scaffolds with different concentrations. The values can more intuitively reflect that when the chitosan concentration increases to 4%, the Young's modulus value of the scaffold in the elastic stage is the best, and the tensile strength is the best. With the scaffold (PCL/Gelatin)

previously used in the vagina on a chip, Angel obtained a Young's modulus value of 0.6MPa. Compared with chitosan/polyvinyl alcohol, it can be concluded that the effect of the scaffold spun in this project is much greater than PCL/Gelatin.

Figure 31 shows the Young's modulus measurements of 87 women by Huang (2023) et al., including 34 women of childbearing age and 53 postmenopausal women. Comparing the Young's modulus in Figure 31 with that of chitosan/polyvinyl alcohol scaffolds, converted to the same units, reveals that the electrospun nanoscaffold has a much higher Young's modulus than vaginal tissue.

Name	Young's modulus(GPa)	Tensile strength(MPa)	Elongation at break (%)
CS2/PVA10	0.252	6.33	7.9
CS3/PVA10	0.55	12.71	9.2
CS4/PVA10	0.846	17.74	8.7
CS5/PVA10	0.297	7.87	9.5

Figure 30. Strain-stress data at four chitosan/polyvinyl alcohol concentrations.

Table 1 VWT and Young's modulus of premenopausal versus postmenopausal women

From: Assessing vaginal wall indexes in premenopausal versus postmenopausal women by transrectal linear array high-frequency probe

	Premenopausal	Postmenopausal	*P* value
VWT of upper section (mm)	9.37 ± 2.77	6.73 ± 2.09	0.000
VWT of middle section (mm)	8.69 ± 2.05	6.45 ± 1.68	0.000
VWT of lower section (mm)	9.21 ± 2.24	6.70 ± 1.98	0.000
Young's modulus (Kpa)	20.81 ± 6.36	18.52 ± 5.27	0.073

Figure 31. Young's modulus of vaginal wall in premenopausal and postmenopausal women(Huang, 2023).

3.7.4 Comparative analysis of FTIR

It can be seen from the FTIR analysis (Figure 32) that the strong peak at 3438 cm^{-1} is an association peak formed by the overlapping stretching vibrations of V(-OH) and V(-NH_2); the double peaks at 2977 and 2878 cm^{-1} are V (-CH_3) and V(-CH_2) stretching vibration absorption peaks. The absorption intensity of these two peaks is relatively strong and the peak shape is steep, indicating that the degree of deacetylation of this sample is relatively high. 1515 cm^{-1} is the characteristic absorption peak of the amide

band, and 1590 cm^{-1} is the deformation vibration absorption peak of δ ($-NH_2$). 1460 cm^{-1} is the deformation vibration absorption peak of δ ($-CH_2$), and 1379 cm^{-1} is the deformation vibration absorption peak of δ ($C-CH_3$), indicating that there are still a certain number of acetyl groups in the chitosan molecule. 1259 cm^{-1} is the deformation vibration absorption peak of δ (-OH). 1152 and 897 cm^{-1} are the characteristic absorption peaks of the β -glycosidic bond of chitosan. 1090 and 1036 cm^{-1} are V(C-O) stretching vibration absorption peaks. 664 cm^{-1} is the crystallIization sensitive peak of chitosan. On the other hand, the V(-OH) stretching vibration peak of polyvinyl alcohol should broaden at 3200~3700 cm^{-1}, and extend to the low wave number to the methylene and methine stretching vibration absorption peaks at 2945 cm^{-1}. It shows that there are a large number of hydrogen bonds within and between molecular chains. 1422 cm^{-1} is the deformation vibration absorption peak of δ ($-CH_2$), and 1330 cm^{-1} is the deformation vibration absorption peak of δ (-OH). 1094 cm^{-1} is the V(C-O) stretching vibration absorption peak, and 1142 cm^{-1} is the crystallization peak of polyvinyl alcohol.

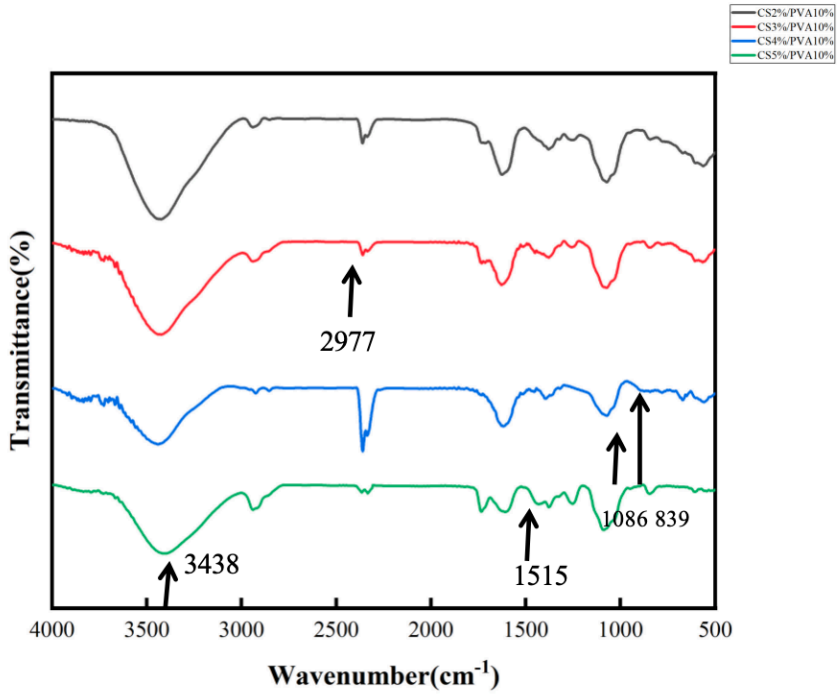

Figure 32. FTIR results of different concentration CS/PVA membrane.

On the other hand, after adding PVA the stretching vibration peaks of V(-OH) and V(-NH_2) of CS at 3427 cm^{-1} broadened and moved toward lower wave numbers, indicating the formation of strong hydrogen bonds between CS and PVA. Due to the effect of hydrogen bonding, the absorption peak of the functional group is shifted, and the characteristic absorption peak of δ(-NH_2) at 1590 cm^{-1} also weakens. This is due to the addition of PVA, which reduces the proportion of amino groups. In addition, the characteristic absorption peaks of β-glycosidic bonds of chitosan at 1152 cm^{-1} and 897 cm^{-1} almost disappeared, and the crystallization sensitive peak of chitosan at 664 cm^{-1} was also greatly weakened. This shows that the addition of PVA destroys the regularity of CS molecular chains and reduces its crystalllinity. The infrared spectra of the blend membranes of three different proportions are similar, and there are no new absorption peaks compared with the pure components, indicating that the blending of the two is only physical mixing, but due to the formation of hydrogen bonds between the two polymer chains, so that the combination of the two is very cLose.

3.7.5 Comparative analysis of XRD

Figure 33 shows the XRD spectra of CS, PVA and four different proportions of electrospun membranes. It can be seen from Figure 33 that CS is a low crystalline polymer with strong diffraction absorption peaks at 2 θ = 9.7. and 19.9 , and a weak diffraction absorption peak at 2 θ = 29.5. PVA is a highly crystalline polymer with a strong diffraction absorption peak at 2 θ = 19.6, and weaker diffraction absorption peaks at 2 θ = 11.6 and 29.5, respectively. If there is no interaction between Cs and PVA molecules in the electrospinning membrane, or the interaction is very weak, the diffraction peak of the membrane is a simple superposition of the crystallization peaks of the two components. However, it can be seen from Figure 33 that as the Cs content increases, the intensity of the crystallization peak of PVA at 2 θ of 19.6 gradually weakens and broadens, which is consistent with reports in the literature.

This further shows that there is a strong hydrogen bonding interaction between the two components in the CS/PVA blend membrane. It is precise because of this interaction that the original crystal structure of PVA is changed, thereby reducing the crystallinity.

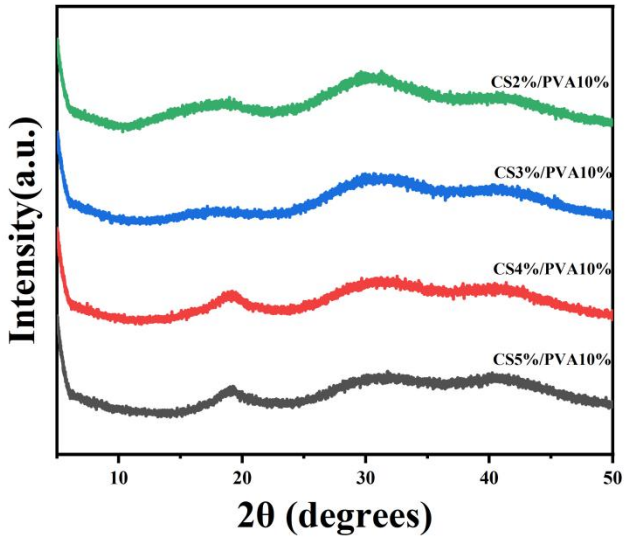

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Figure 33 .XRD curve of different concentrations CS/PVA electronspun membrane.

Chapter 4.Fabrication process of vagina on a chip and platform

4. Fabrication process of vagina on a chip and platform

The design of organ-on-a-chip is a complex process, including the restoration and testing of the microenvironment of specific organs in the human body. In other words, it is an important model platform, providing a way to conduct simulation studies in relatively simple organisms in vitro and in vivo of extremely complex organisms. In a sense, the modeling process is an abstraction of things themselves in order to understand things, abstracting complex practical problems into a relatively simple physical model that contains the core content of practical problems. The built model is used to describe the overall and local relationships in the system. This means that the design of the chip platform needs to meet the needs of experimental operations, so as to ensure the effective combination of chitosan/polyvinyl alcohol scaffold and chip. Based on this background, Chapter 4 mainly developed and designed a simplified upper and lower vaginal chip, which has a cross-type liquid flow channel to ensure the supply of culture medium and nutrients when VK2/E6E7 cells are cultured in vitro. In addition, the chip design process was summarized, including the chip size parameters, manufacturing process and surface modification treatment, and the theoretical basis was expanded as the experimental basis, thus providing a new pathological model for the design of the vagina on a chip platform.

On the other hand, in view of the fact that the scaffold is prone to curling and wrinkling, a unique experimental method combining the chip platform and the scaffold was developed, so that the scaffold is placed in the center of the upper and lower layers of the chip, thereby ensuring that the cells have a stable carrier when cultured in vitro.

4.1 The problem of vagina on a chip aims to solve

Bacterial Vaginosis is a disease caused by the replacement of normal commensal lactobacilli that produce lactic acid and hydrogen peroxide in the vagina which produce a protective layer on the vaginal epithelial cells, by an overgrowth of a large number of anaerobic bacteria (e.g. Gardnerella vaginalis, prevotella, Mobilus and other bacterial vaginosis-related pathogens). A prominent feature is the presence of polymicrobial biofilms on vaginal epithelial cells. It can be seen that the pathogenesis of bacterial vaginosis mainly comes from mixture of different bacterial infections. For the construction of vagina on a chip, how to simulate the microenvironment and tissue structure inside the female vagina is the key to the design of vaginal chip platforms.

The cell walls in the vagina are typically full of folds increasing the surface area and allow the epithelium to stretch as required, i.e. it is very elastic, this means that the in vitro model need certain mechanical properties to simulate the mechanical environment inside the vagina. Secondly, the structure of the chip needs to meet the environment for culturing vaginal cells, including vaginal epithelial cells and connective tissue cells. As a consequence, the vagina on a chip design based on microfluidic chips needs to establish fluid channels to supply cell culture medium and other nutrients needed by vaginal cells, and build a mechanical environment of vaginal tissue on the chip to simulate the characteristics of internal muscles.

In addition, the design of the chip channel needs to ensure the fluidity of the liquid so that vaginal epithelial cells can grow and differentiate normally on the scaffold. In other words, the simulation of the vagina on a chip needs to be simplified to ensure that the chip can be tightly assembled with the scaffold while the external system controls the flow and exchange of liquid. Especially in the assembly of the scaffold and the chip, due to the characteristics of the chitosan/polyvinyl alcohol film, the cross-linked scaffold will quickly wrinkle after leaving the liquid environment and cannot form a complete shape. This is a difficult problem to solve for chip assembly.

On the other hand, the placement of the scaffold on the chip needs to ensure its stability, which is crucial for cell growth. The growth space of vaginal epithelial cells requires a stable microenvironment to support cell growth and differentiation, which requires that the position of the scaffold on the chip needs to be fixed by special methods. For this special method, the first thing to explain is that due to the characteristics of the scaffold itself after being made by electrospinning, it has high elasticity and wrinkles, which increases the difficulty of assembling the scaffold and the chip. Therefore, before assembling the scaffold and the chip, the scaffold is placed in a PBS solution and transferred to the central position of the lower chip channel using sterile tweezers. At this time, it will be observed that the scaffold quickly wrinkles and curls after leaving the liquid environment. Using a pipette to quickly draw a small amount of PBS liquid into the chip channel to observe the morphology of the scaffold. Besides, using sterile tweezers to grab the edge positions of the scaffold around the curled part and unfold it, and stably

fix it in the central position of the chip channel. It is worth noting that this process cannot destroy the original morphology of the scaffold, including the rupture of the scaffold, which affects the in vitro culture of Vk2/E6E7 cells.

4.2 Vagina on a Chip Design Solution

In this part, fabricating the appropriate structure of the chip is the key to creating the model. As mentioned above, this project aims to solve the relevant vaginal disease such as bacterial vaginosis, thus the structure of the chip needs to simulate the approximate vaginal microvironment. Firstly, Solidworks was used to design the structure of the chip which can satisfy the experimental requirement. Figure 34 shows the design diagram of the vagina on a chip, it can be divided into two layers and integrated together to form the whole vaginal platform. The upper and lower layers of the chip can be assembled by meshing together through the surrounding circuit holes. The number of layers in the microfluidic chip simplifies the fluid flow path through the chip, and this simplified fluid path reduces the risk of liquid leakage from the chip. This effectively ensures the feasibility of connecting the microfluidic system to create an in vitro culture platform in subsequent experiments.

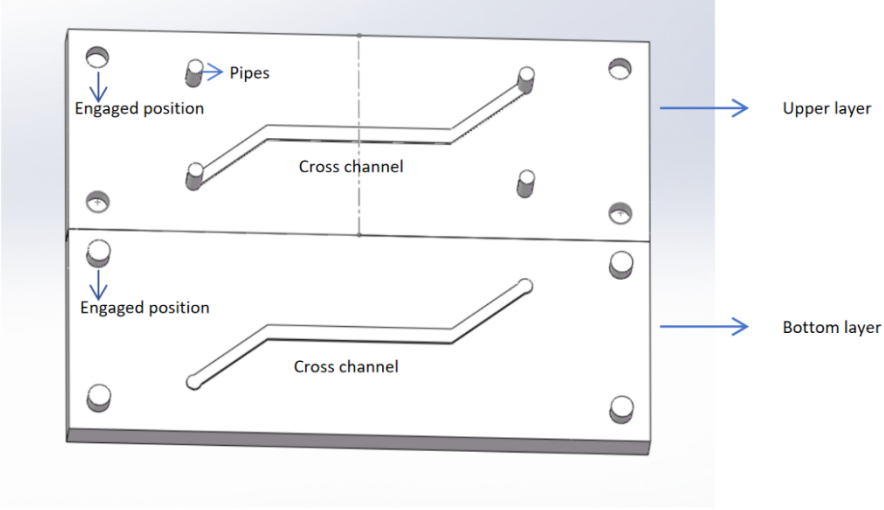


Figure 34. vagina on a chip design diagram

In addition, an electrospun membrane can be placed in the middle of the combination of the upper and lower chips, which is located in the middle of the intersecting fluid channel, ensuring that the liquid flows through the nanofibers while simulating the internal environment of the vagina. The flow of cell culture medium or other liquids through the electrospinning membrane provides a nutrient environment for cell growth, and the

mechanical properties of the electrospinning membrane provide an in vitro basis for cell growth and differentiation. The size of the chip will be described in detail below.

Figure 35 shows the detailed parameter data diagram of the upper and lower layer designs of the chip. The chip is 79 mm long, 29 mm wide, and 5 mm thick in a single layer. The width of the cross channel is 2mm to ensure smooth flow of liquid in the channel. Four circuit holes with a diameter of 4 mm are set at the four corners of the lower layer of the chip to connect to the upper chip. The four circuit holes are 3 mm away from the edge of the chip and the cross channel protrudes 0.5 mm thick on the chip. At the same time, the ends of the cross channel are respectively provided with an embedding port for connecting to an external liquid control system, and the diameter of the embedding port is 1 mm, so as to ensure that the pressure between the liquid and the scaffold when it flows in the chip will not cause the scaffold to shift in position (Cho, 2023). The length of the cross channel in the middle of the chip is 25 mm.

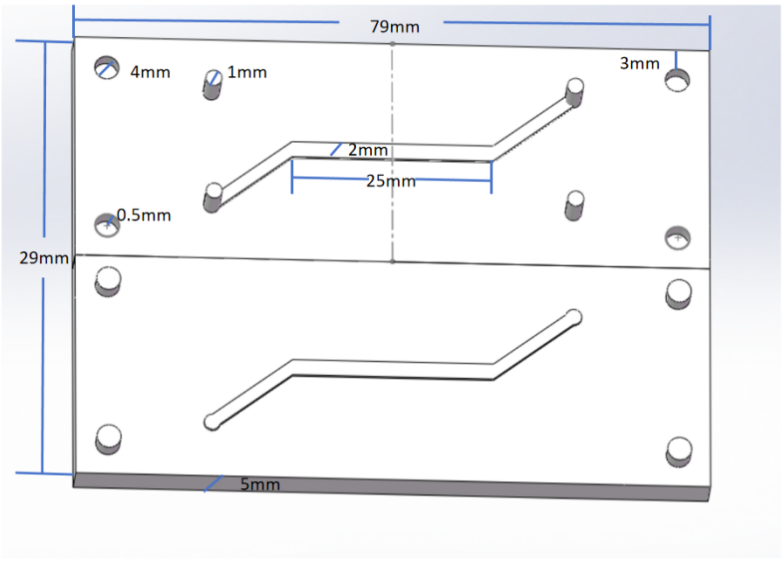


Figure 35. Parameter data of chip design

The overall design parameters of the chip need to ensure the normal circulation of liquid in the chip, and the pressure and shear force generated cannot affect the placement of the bracket in the middle position. Excessive liquid pressure will cause

the bracket to deviate, thus affecting the growth of cells; while too little liquid pressure will make the liquid flow too slowly, making it impossible to circulate and exchange the culture medium and nutrients in a timely and effective manner. On the other hand, the design of chip parameters need to consider the feasibility of actual production and operation.

Consequently, the simulation of different human tissues by organ on a chip is based on real organ characteristics, so that the designed chip structure can maximize the simulation effect. The female reproductive system is a complex system, which includes organs such as the cervix, uterus, fallopian tubes and vagina to form the reproductive system. For the vaginal organs, smooth liquid control and peristalsis of muscle tissue are key factors in its bionics. As shown in the design diagram above, the double-layer chip simplifies the channels for liquid circulation, and the cross channels can realize unidirectional and bidirectional liquid flow. This has a promoting effect on the subsequent connection to the external liquid control system. The high ductility and elasticity of the chitosan/polyvinyl alcohol scaffold simplifies the assembly process when the scaffold is combined with the chip. Based on the above description of the chip design, it clearly shows the structural characteristics of the chip, thus providing a convenient basis for the operation of subsequent experiments. In the following chapters, the fabrication, integration and testing process of the chip will be analyzed and explained in detail.

4.3 Fabricating process of vagina on a chip

Based on the design and structure of the vagina on a chip, the manufacturing process of the chip can be mainly divided into two parts, including 3D printing technology to make the chip model and using PDMS material to manufacture the chip. The application technology of 3D printing on microfluidic chips is becoming mature. Its efficient and fast speed and precise and convenient operation enable the printed chips to fully achieve the purpose of targeted design. Verowhite is an opaque polystyrene resin material, which is widely used in the field of bioengineering to make molds.

4.3.1 Fabricating of the Chip Model

After the structure was designed in Solidworks, an STL file was produced. VeroWhite(liquid), a photosensitive resin commonly used in 3D printing, is widely used in the development of molds for medical products, primarily due to its high precision and strong rigidity. The vagina on a chip mold was primarily produced using an Object 30 Pro, importing SolidWorks designs for printing. The laser spotting under the control of the light source reacts to the Verowhite material, so that the light curing reacts to the specific position under the parameters of the chip design drawing, thereby completing the chip construction process under the 3D printer. After printing, the collected chip need to be calibrated, including the measurement of the size to ensure the integrity and accuracy of the parameters again. As mentioned in the chip design section above, the upper and lower chips are complete cubes with no suspended structure in the middle, so the mold can be made without using a support structure during the 3D printing process. After the chip is calibrated, it needs to be post-processed. The post-processing of the mold 3D printing often uses sandpaper to polish the mold surface. After the mold is printed, placing it in pure water and let it stand for 5 minutes, then use sandpaper (No. 400 and 600) to gently polish the mold surface to remove fine particles and impurities on the surface. Figure 36 shows the 3D printed mould.

On the other hand, the finished mold was also measured to determine the accuracy of the dimensions compared to the design. The actual mold measured 78.7mm in length, 29.1mm in width, and 5mm in thickness. The accuracy of the remaining mold components was within 0.03mm. The results of the actual experiment showed that this 0.03mm accuracy error was well within controllable limits.

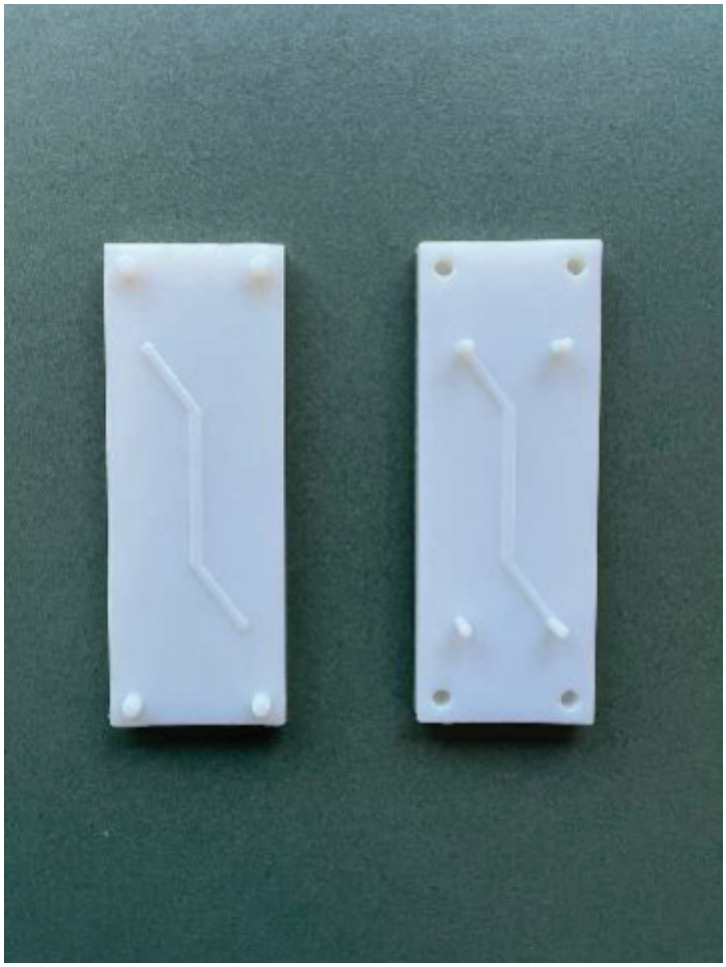


Figure 36. The 3D printed mould

The chip model made by 3D printing technology is bonded with the mould casted by PDMS material, which provides an engineering basis for the next step of chip production.

4.3.2 Preparation process of PDMS chip

PDMS material stands for polydimethylsiloxane, which is a kind of organic silicon material with good biocompatibility and flexibility, and is widely used in bioengineering, specifically in the lab on a chip/organ on a chip fields. Chips made from PDMS are transparent, so that the flow of liquid in the chip and the related reaction process can be effectively observed. PDMS is a material that is easy to modify, and its surface properties can be improved by physical or chemical means. As shown in Figure 37, the functional group structure of PDMS is shown, and the segmented structure of its chemical formula is in the middle. The high and low values of n represent the state of PDMS. When the n value is low, it is in liquid form; when

the n value is high, it is in semi-solid form. The cross-linked PDMS property is a hydrophobic monomer, common solvents find it difficult to wet the surface of PDMS, and liquid droplets will not diffuse on its surface, resulting in the PDMS surface being able to adsorb hydrophobic substances in the liquid. On the other hand, the chemical properties of the PDMS surface can also be changed by plasma modification, so that it changes from a hydrophobic material to a hydrophilic material. The essence of plasma modification is to change the oxidation and change the PDMS surface, so that silanol is generated on its surface, thereby increasing its hydrophilicity. This method effectively resists the adsorption of hydrophobic and negatively charged molecules on the PDMS surface. In addition, it does not allow water, glycerol, methanol or ethanol to penetrate and continuously deform, regardless of whether the PDMS surface is plasma oxidized. Therefore, PDMS can be used with these fluids without worrying about microstructure deformation.

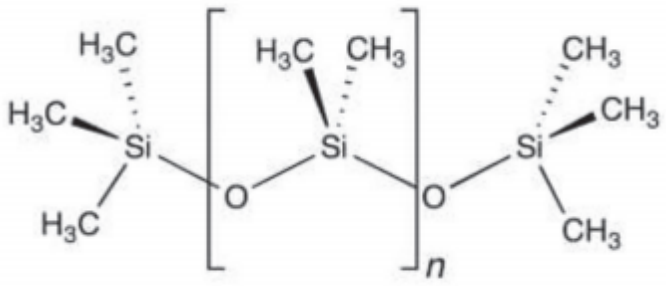


Figure 37. PDMS functional group structure. (Eduok, 2017)

In summary, PDMS material is transparent at optical frequencies, which greatly facilitates the visual observation of its internal state or objects under a high-power microscope. Its inherent biocompatibility ensures the feasibility of cell growth on its surface, thereby ensuring the smooth in vitro cell culture of organ chips. On the other hand, in actual experimental operations, PDMS is easy to shape and can maintain a plastic state, while ensuring air permeability and sealing. Next, the experimental operation of the PDMS chip will be described in detail.

After the designed moulds are printed, the next section was to manufacture the chip. Firstly, preparing the chemical mixture solution which applied into the manufacture process. PDMS material used in the solution, the ratio of these two chemicals SYLGARD 184 Silicone Elastomer Base and SYLGARD 184 Silicone Elastomer curing Agent is 1:10 (mL). Normally, choosing 2 mL for the SYLGARD 184 Silicone Elastomer curing Agent and 20 mL for the SYLGARD 184 Silicone Elastomer to acquire the PDMS mixture solution of 20mL and stir the mixture by hand for 2-3 minutes. Then, putting the PDMS mixture solution in the desiccator. The aim of desiccating process was to eliminate bubbles that formed when blending the two chemicals together by hand; bubbles can generate incomplete chip moulding. The desiccator is attached to a vacuum air pump which when turned on, removes any residual gas from the PDMS mixture. With degassed PDMS mixture, the next section is pouring the solution into the settled mould slot. As Figure 38 shows, putting the designed mould into the groove on the settled slot and utilizing four screws to fix the two slot together. After confirming that the mould slot is tightly fixed, pouring the PDMS mixture solution into the groove evenly until the solution is paving the groove surface. On the other hand, there are holes on the four corners of the groove surface which can integrate the two various layers to one chip. Therefore, after pouring the PDMS solution into the groove, a needle was used to eliminate the bubbles remaining in areas such as sharp corners. Otherwise, the bubbles in the mould lead to chip model incompletely.

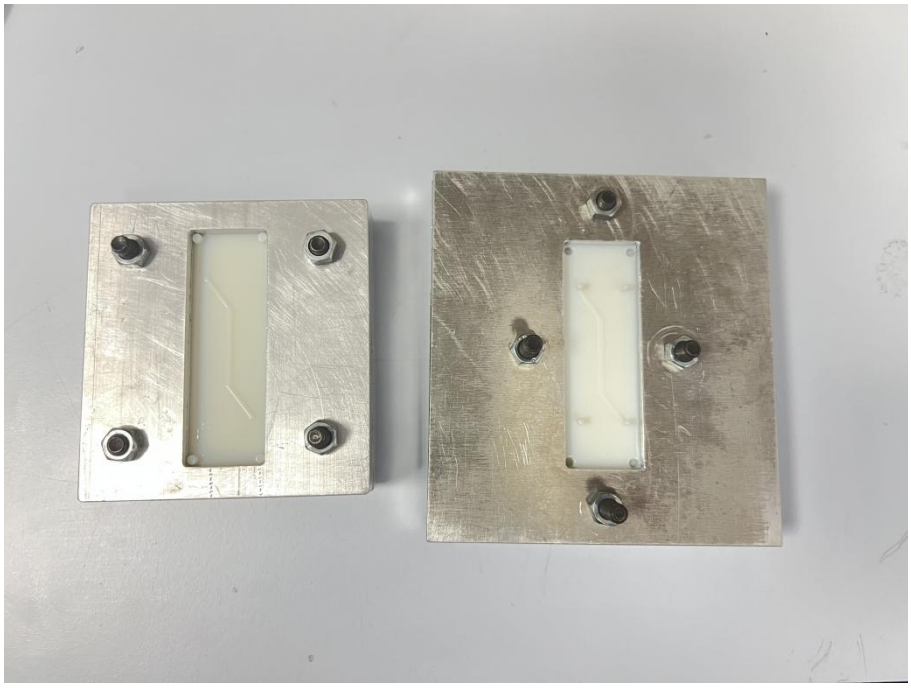


Figure 38. The integrate process of chips and mould

With the PDMS poured, they were placed in an oven at 50℃ for 2-3 hours. The moulds were taken out of the oven and cooled for 30 minutes to acquire the chip model. As the description of the method of fabricating chip model, set two parallel fluid channels in the channel of the chip which can make the liquid flow smoothly through the channel. In other words, the flow method and the micro channel network determine the function of the chip to a large extent. The flexible micro channel network design is a key to the operation design of the basic unit of the microfluidic chip. For these basic operating units, they rely on micro channel network connection and the key component for smooth operation of the network connection is the micro-pump micro-valve. The high-performance micro-pump and micro-valve is also necessary for the formation of a large-scale integrated microfluidic chip laboratory. As a consequence, the designed chip provides the connected channel and network to make liquid flow smoothly in the whole chip and establish the microenvironment of the vagina.

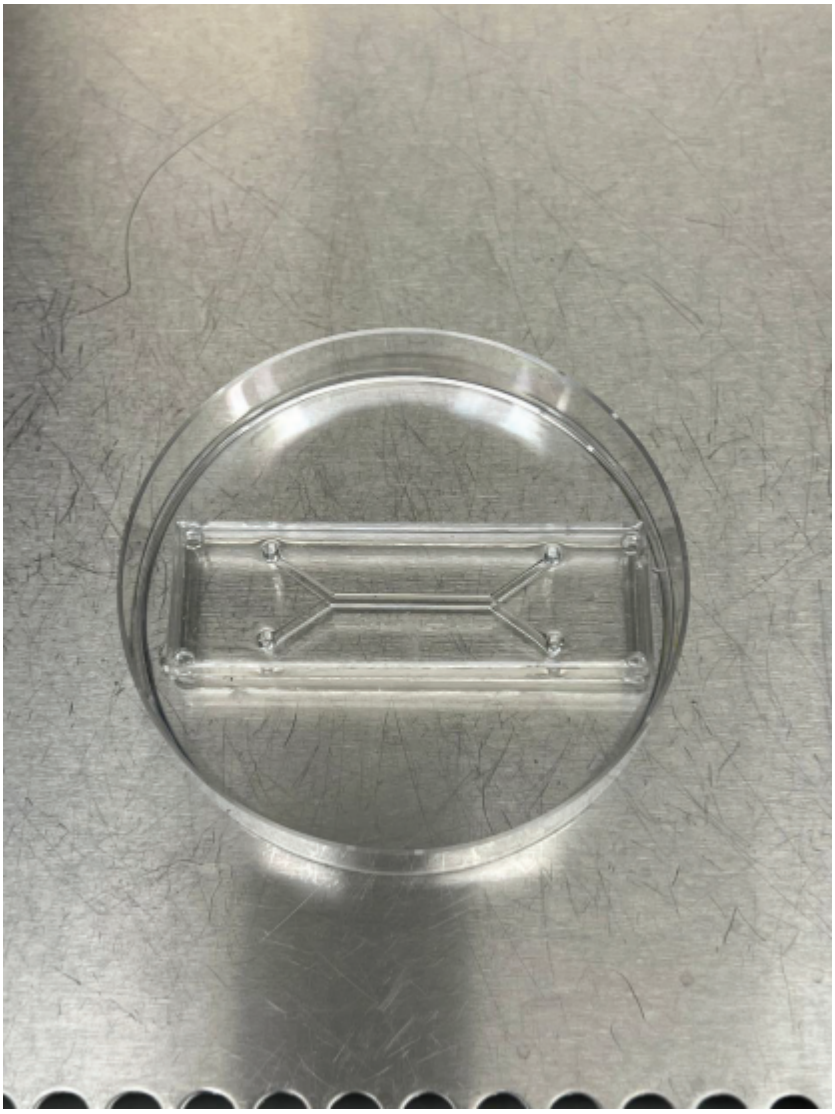


Figure 39. The PDMS of vagina on a chip

The transparent PDMS chip with upper and lower layers shown in the picture (Figure 39) is the microfluidic chip structure of the vagina on a chip platform. It can be clearly seen that the simplified chip structure ensures the smooth flow of liquid, while the reserved sacffold placement position in the middle also provides growth space for the growth of vaginal epithelial cells.

4.4 Assembly of chitosan/polyvinyl alcohol scaffold and chip

4.4.1 Chip assembly and modification

The modification of PDMS material is an important step in the combination of the upper and lower layers of the chip. The first reason is that the cured PDMS has low surface energy and poor surface wettability, which makes it difficult for liquid to flow

in the PDMS micro channel, so it is necessary to treat the surface of PDMS to improve its surface wettability. Secondly, the modification can treat the oxygen plasma on the surface of PDMS, so that the chips can be better bonded together.

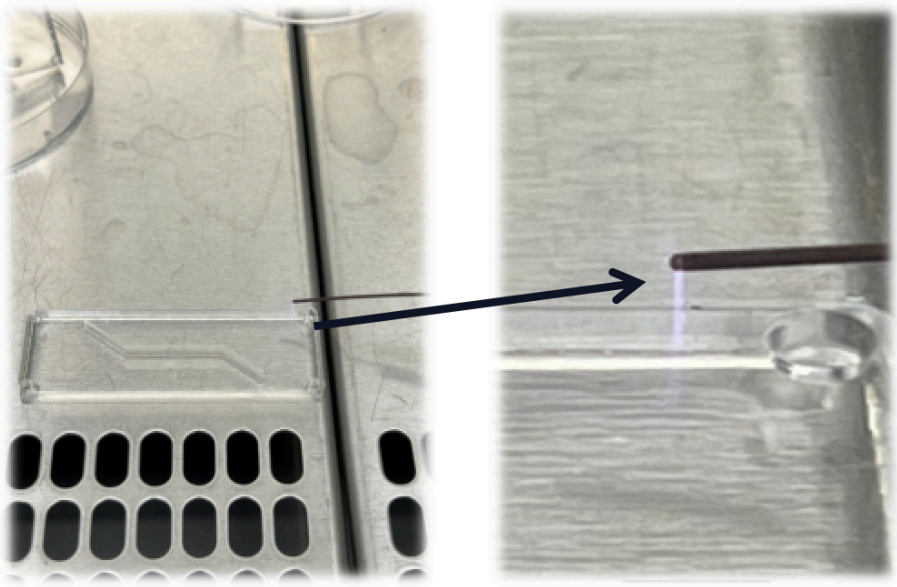


Figure 40. Plasma-modified PDMS surface manipulation

Figure 40 shows the actual process of plasma modification of the PDMS chip surface. The right side shows the effect of plasma modification on the PDMS material surface, which will produce visible blue-purple ionized sparks. After modification, the PDMS surface will first become hydrophilic, which is beneficial for subsequent cell culture operations; the second point is that it can improve the viscosity and firmness of the upper and lower chip assemblies, so that the chips can fit better, thereby preventing the leakage of liquid from the chip bonding position, thus affecting the experimental operation and results.

The specific operation of chip surface plasma can be divided into the following three main steps:

① Placed the bonding surface of the upper and lower chips flat on the operating table to ensure that the surface of the chip was clean and free of other contaminants.

② Utilized a handheld plasma modification device and placed the spray position 0.5 cm above the chip for modification. The bonding surface of each chip was plasma

modified for 120 seconds. It should be noted that there were different circular hole joints and external pipes on the chip bonding surface. Plasma sparks are corrosive to a certain extent.

③ The modification of the chip surface by plasma sparks needed to start from the edge. This was to ensure that the chip can be more tightly sealed when the upper and lower chips were combined, so as to prevent the leakage of liquid from the edge of the chip when cells were cultured on it.

For example, after modifying the edge of the chip for a circle, move to the center of the chip from the outer circle to the inner circle. The benefit of this operation is that it can ensure that every part of the chip surface can be covered by plasma sparks to ensure the integrity of the combination of the upper and lower chips. As a consequence, Plasma oxidation is a common modification method for PDMS materials, which effectively changes its surface from hydrophobic to hydrophilic, so as to carry out subsequent biological experiments. Related studies have shown that the modification of PDMS and other related materials can improve the adsorption of cells on the chip surface, thereby promoting cell growth and differentiation, thereby improving the feasibility of in vitro cell culture.

4.4.2 Integration of chitosan/polyvinyl alcohol scaffold and chip

The chitosan/polyvinyl alcohol scaffold appears as a white membrane on the tin foil, which is the initial state of the scaffold after spinning. The cross-linked membrane changes the functional group structure on its surface and makes it hydrophobic, thus insoluble in liquid. The assembly of the scaffold and the chip is a complex process. It is necessary to ensure that the scaffold is effectively placed on the chip and will not be offset, and it is also necessary to ensure that the growth of cells on the scaffold can simulate the physiological microenvironment inside the vagina. Based on this background, the assembly operation of the chitosan/polyvinyl alcohol scaffold and chip will be explained and illustrated in detail below.

Firstly, the scaffold needs to be pretreated to ensure the sterility and integrity of the cells growing on the scaffold. After cross-linking, the chitosan/polyvinyl alcohol scaffold is cut into a rectangular film with a length of 2.5 cm and a width of 1 cm, and

then the film is completely peeled off from its carrier foil for sterilization. The sterilization operation is to rinse the film in 70% ethanol three times for 5 minutes each time; then rinse it in PBS liquid three times for 5 minutes each time; after rinsing, it is stored in a small amount of PBS liquid. It should be noted that before assembling the scaffold and chip, they should be placed in a UV sterilizer for 1 hour for disinfection and sterilization to ensure the sterility of the scaffold and chip.

Secondly, with the help of tweezers, carefully place the scaffold in the middle of the channel on the surface of the lower chip, and evenly spread it on the surface of the channel to ensure that the scaffold can completely cover the upper position of the channel. This operation is an extremely difficult process because the chitosan/polyvinyl alcohol scaffold has good ductility and wrinkle properties. Especially after being separated from the liquid, it will wrinkle into an irregular shape and state and it is difficult to restore it to its intact state in the liquid. Therefore, tweezers are needed to carefully extend the scaffold, and this operation can be assisted by a small amount of PBS liquid to help restore the shape of the scaffold. After the scaffold is laid flat, PDMS liquid needs to be applied to the edges of the lower chip to ensure the sealing of the upper and lower chips. The PDMS liquid itself has a certain viscosity, and when the upper and lower chips are attached, it will not contaminate the chip surface or damage the surface structure.

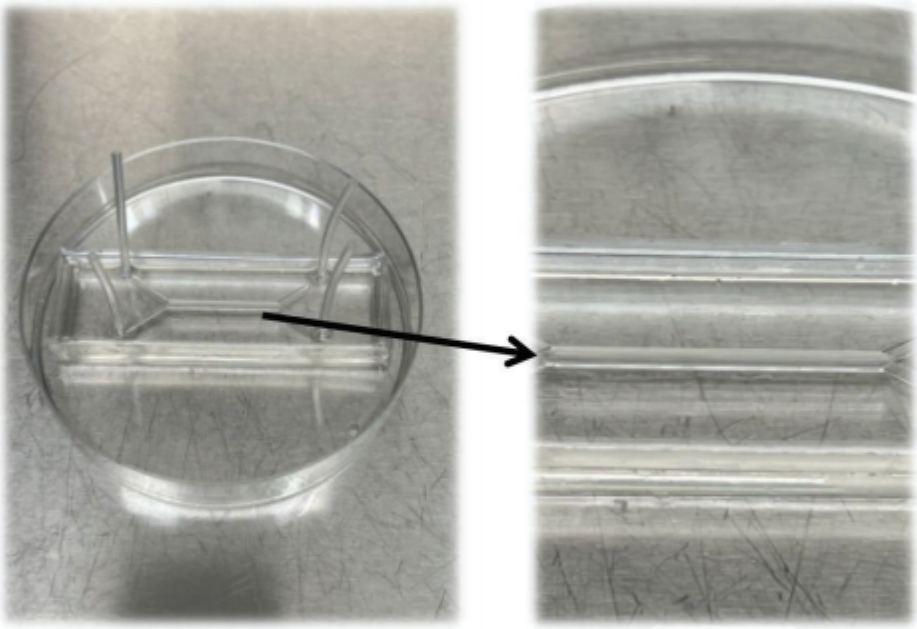


Figure 41. Assembly of chitosan/polyvinyl alcohol scaffold and chip

Figure 41 shows the actual effect of the integration of the chitosan/polyvinyl alcohol scaffold and the chip. The enlarged part on the right shows the state of the scaffold in the chip. It can be clearly seen that the chitosan/polyvinyl alcohol scaffold is a translucent film placed in the middle of the chipIs cross-shaped channel and completely covers its space.

The integrated chitosan/polyvinyl alcohol scaffold and chip construct a platform for the vagina on a chip, which can effectively ensure the fluidity of the chip and the integrity of the chip seal under the control of the external system. The fluid control system verifies that the liquid flows smoothly in the integrated vaginal chip, and the pressure and fluid shear force it generates will not affect the position of the scaffold, thereby affecting the offset of the scaffold. On the other hand, the integrated vagina on a chip provides complete growth conditions for the growth of vaginal epithelial cells. The ductility of the chitosan/polyvinyl alcohol scaffold and its good mechanical properties can effectively simulate the peristaltic characteristics of the muscle tissue inside the vagina, which is undoubtedly more conducive to the growth of vaginal epithelial cells. The fluid control system simulates the flow characteristics of the liquid inside the vagina and provides nutrients for cell growth. In summary, the

construction of the vagina on a chip platform has created a new three-dimensional culture simulation platform for the growth of vaginal epithelial cells, which allows real human vaginal epithelial cells to be cultured in vitro while also creating the feasibility of further studying the implantation of related pathogens.

Chapter 5. Evaluation of vagina on a chip

Biocompatibility

5. Evaluation of vagina on a chip Biocompatibility

The in vitro culture of vaginal cells is limited by the growth characteristics of the cells themselves, and the growth of these cells were found to be more challenging than other immortalized cell lines. The in vitro culture of vaginal epithelial cells has great uncertainty, and their growth time and passage cycle are not as regular as other cells, which undoubtedly increases the difficulty of in vitro culture of vaginal epithelial cells. Based on this background, this chapter explores in vitro culture growth and differentiation of vaginal epithelial cells and summarizes appropriate culture methods. On the other hand, the above integration of chitosan/polyvinyl alcohol scaffolds and chips determines the feasibility of culturing cells on them, and the growth and structural characteristics of cells are also analyzed in this chapter.

The experimental design is mainly divided into the following steps: 1. The appropriate method of in vitro culture of vaginal epithelial cells and the regular cycle of their growth and differentiation were explored, including the subculture cycle and the structural characteristics of cells cultured in vitro; 2. The survival and growth tests were conducted on vaginal epithelial cells grown on chitosan/polyvinyl alcohol scaffolds, and the growth of vaginal epithelial cells on three different nanofiber membranes were explored, thereby verifying the effect of the mechanical properties of the scaffold on cell growth; 3. The fluorescence tracking test explored the structural characteristics of cells growing on the vagina on a chip, and explored whether the cells cultured in vitro have characteristics similar to those in the real human vagina.

5.1 Experimental reagents and equipment

All materials used in biological experiments, including cell culture medium, nutrients required for growth, cell line types and related test reagents will be listed in detail below in Table 4 and Table 5.

Regent Name	Catalog No.	Manufacturer
Keratinocyte SFM	17005042	Thermo Fisher
Penicillin-Streptomycin	15140122	Thermo Fisher
Trypsin (2.5%)	15090046	Thermo Fisher
DMEM/F-12, GlutaMAXⅢ	10565018	Thermo Fisher
DPBS	14190144	Thermo Fisher
HEPES (1 M)	15630106	Thermo Fisher
Fetal Bovine Serum,	10082147	Thermo Fisher
Thiazolyl Blue Tetrazolium Bromide	M5655-100G	Thermo Fisher

Table 4. Experimental materials

Name	Manufacturer
Titramax 100	**Heidolph**
Centrifuge 5804 R	**Eppendorf**
Microplate Reader Elx808	**BioTeK**
Microscope DMi1	**LEICA**
DM4000B Fluorescent Microscope	**LEICA**
OB1 MK4 Pressure controler	**ElveFlow**

Table 5. Experimental equipment

5.2 Experimental methods

5.2.1 Culture of human vaginal epithelial cells

The vaginal epithelial cells used in this project the cell line type is VK2/E6E7, provided by professor Raina Fichorova of Harvard university, in kind. The cells were taken from a 32-year-old woman with endometriosis during vaginal surgery in 1996, and the relevant cell lines were established immortalized and preserved. The human vaginal epithelial cells are different from the vaginal cells of mice or rabbits studied previously. They truly reflect the growth and differentiation of human vaginal cells, and can truly reflect the pathological process of human bacterial vaginosis. The growth of human vaginal epithelial cells is highly uncertain. Different from other epithelial cell lines, the time for attachment and differentiation as well as the required physiological microenvironment are variable. Therefore, this section explores the process of culturing real human vaginal epithelial cells and provides accurate operating steps.

5.2.1.1 Thawing cells

Before culturing vaginal epithelial cells, their culture cycle and passage number need to be determined. Normal cell culture and related biological tests need to be

completed within ten passages of the cells. Too many passages will affect the subsequent growth of the cells, resulting in inaccurate results of subsequent biological tests. Therefore, this experiment selected vaginal epithelial cells that were stored in a liquid nitrogen chamber and passaged four times.

1. After the vaginal cells were taken out of the liquid nitrogen chamber, they were placed on a rack and put into a water bath for thawing. The water bath mperature was 37℃ and the thawing time was 5 minutes.
2. Thawed vaginal epithelial cells (usually 1 ml) were carefully piped into a 15 ml centrifuge tube, then 9 ml of Dulbecco's Modified Eagles Medium added. The purpose of this step is to quickly provide the thawed cells with the corresponding nutrients and reduce toxicity of the DMSO they are frozen down in, thereby stimulated the growth activity of the cells. It should be noted that the DMEM medium needs to be added with 5% Horse serum nutrients.
3. Placed 10 ml of vaginal epithelial cells and DMEM culture medium in an ultracentrifuge, set the centrifugal speed to 500 RPM and centrifuge for 5 minutes. Vaginal epithelial cells and culture medium have different densities, which will separate outward in the radial direction under the action of centrifugal force, and the precipitate of vaginal epithelial cells can be collected.
4. Used a pipette to remove the DMEM culture medium on the top of the centrifuge tube. It should be noted that the operation should be carried out slowly to avoid disloding the vaginal epithelial cell pellet at the bottom. Added keratinocyte serum-free medium dedicated to vaginal epithelial cells. The proportion of SFM added was generally determined by the desired dilution multiple. The author explored the most suitable ratio of vaginal epithelial cells after thawing. Firstly, added 1 ml of SFM culture medium to the centrifuge tube and mix it evenly with the vaginal epithelial cell pellet, and then transfer it to a T-75 flask containing 9 ml of SFM culture medium. The growth environment of vaginal epithelial cells at this dilution is the best.
5. The diluted vaginal epithelial cells were placed in an incubator and cultured for 72 hours to observe their growth. Generally speaking, 72 hours of culture will not allow the vaginal epithelium to adhere to the wall, which was also the difficulty in distinguishing it from other adherent cells. Vaginal epithelial cells need a longer time to achieve adhesion.

5.2.1.2 vaginal epithelial cell culture

As mentioned above, vaginal epithelial cells generally need more time than other adherent cells to achieve adhesion. Therefore, during the culture and maintenance of cells, the author has explored and summarized the most suitable method to make vaginal epithelial cells completely adhere to the wall. Observe the growth of cells 72 hours after thawing. At this time, the cells are still in a suspended state, and do not replace the culture medium for the cells in the flask until some cells are observed to be attached to the wall, and then replace the culture medium. The time period for vaginal epithelial cells to achieve adhesion is 7-10 days. It should be noted that although the culture medium in the flask does not need to be replaced in the early stage, the growth of cells should be observed every two days.

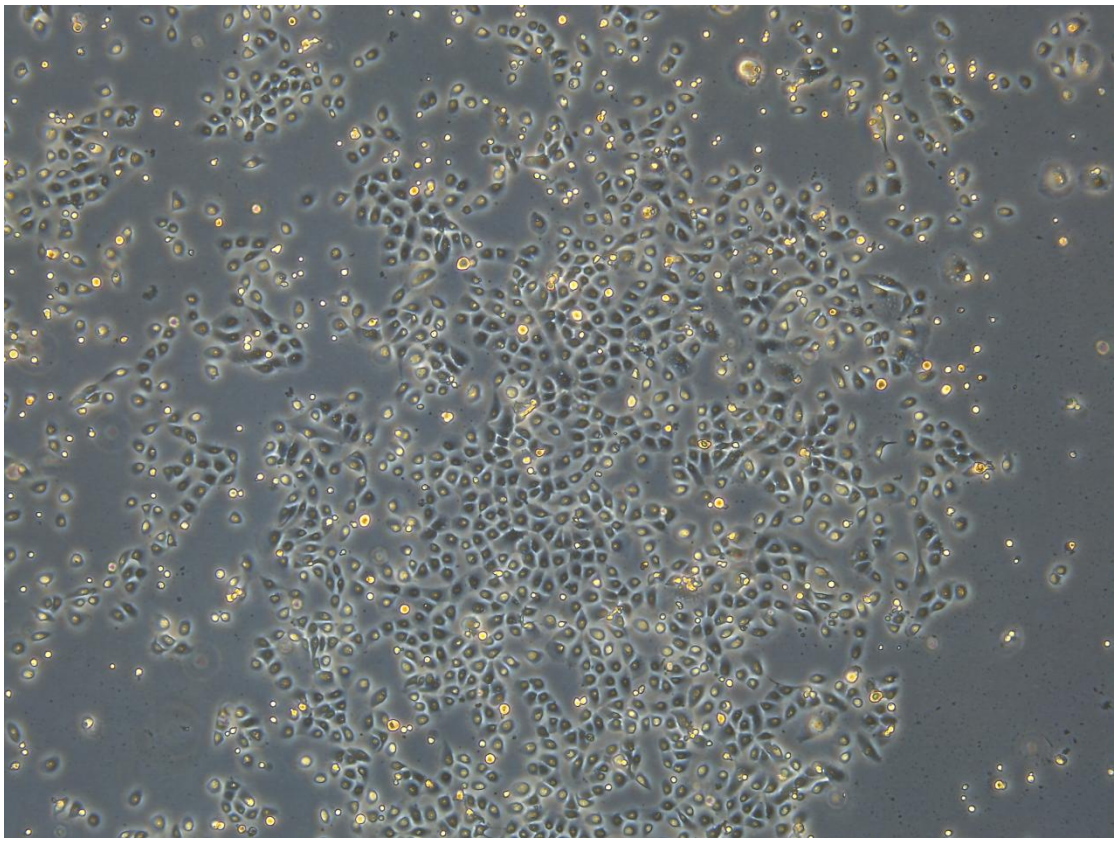


Figure 42. Vk2/E6E7 cells undergoing initial adherent growth

Figure 42 shows the growth of vaginal epithelial cells that are completely attached to the wall and fused under a microscope, which can clearly show the morphological characteristics of vaginal epithelial cells and the fusion between cells. Its shape is irregularly round, often polygonal, similar to a folded form, and after attachment and differentiation, it appears like fish scales as a whole. This morphology proves

that vaginal epithelial cells grow well and have a normal cell differentiation rate. On the other hand, the cell confluence needs to be observed during cell culture, and the cells need to be subcultured when the cell confluence is 80%. The purpose of cell subculture is to make the cells grow faster after attachment, so that the cells need more space to maintain normal growth. The cell subculture time varies according to different cell dilution multiples. The vaginal epithelial cell subculture cycle under this dilution multiple is 8-10 days.

5.2.1.3 Subculture Vk2/E6E7 cells

Cell passaging is a common operation in the cell culture process and is also a very important step. Cells after passaging often show better growth characteristics. The passaging operation steps for Vk2/E6E7 are as follows:

1, After the cell suspension in the T-75 flask was completely aspirated with a pipette, 5 ml of PBS liquid was added to wash the flask. The purpose of this step was to ensure that there was no residual cell suspension in the flask and to clean it up. It should be noted that during the PBS cleaning process, the PBS liquid in the flask can be carefully rotated so that it can fully contact the surface of the cell adhesion, thereby ensured a more thorough cleaning.

2, After PBS washed, aspirated the PBS liquid; then aspirated 5 ml of trypsin into the flask to digest the vaginal epithelial cells. The cleavage site of trypsin was the carboxyl terminal peptide chain of the two residues of Lys or Arg in the peptide chain. By degrading proteins at specific positions, the proteins on the cell membrane and the junction of the culture dish were degraded, thereby separated the two. At this time, the cells became spherical due to the tension of their own internal cytoskeleton and the surface tension of the culture medium. It should be noted that the trypsin cell digestion time should not be too long, otherwise the growth condition of the cells will be poor after plating. After added trypsin, moved the T-75 flask to the incubator and incubated for 10 minutes, then took it out and observed the digestion of the cells.

3 , Observed the digestion of cells under a microscope. If most of the cells were not completely digested, they need to be moved to the incubator for digestion. If a small number of cells were not completely digested, gently tapped the sides of the flask to make them fall off slowly under the action of external vibration. After trypsin digestion, added 10 ml of DMEM medium to deactivate the trypsin and transfered them to a centrifuge tube for centrifugation. Centrifuge at 500 rpm for 5 minutes to collect the cell pellet. Aspirated the DMEM medium on the top of the centrifuge tube and leaved the remaining cell pellet at the bottom.

4. Added vaginal epithelial cell culture medium to the centrifuge tube. The amount of culture medium added as described above was the dilution multiple, and the dilution multiple was adjusted according to the desired ratio. After added the culture medium, observed the cells. At this time, the cells appeared round and suspended in the flask, and then moved to the incubator for culture. It should be stated that the adhesion time of cells after subculture was accelerated. Unlike the adhesion time after thawed, some cells after subculture can achieve adhesion after 48 hours. Therefore, the culture medium of cells after subculture needs to be replaced every 2-3 days to meet the normal growth of cells in the flask.

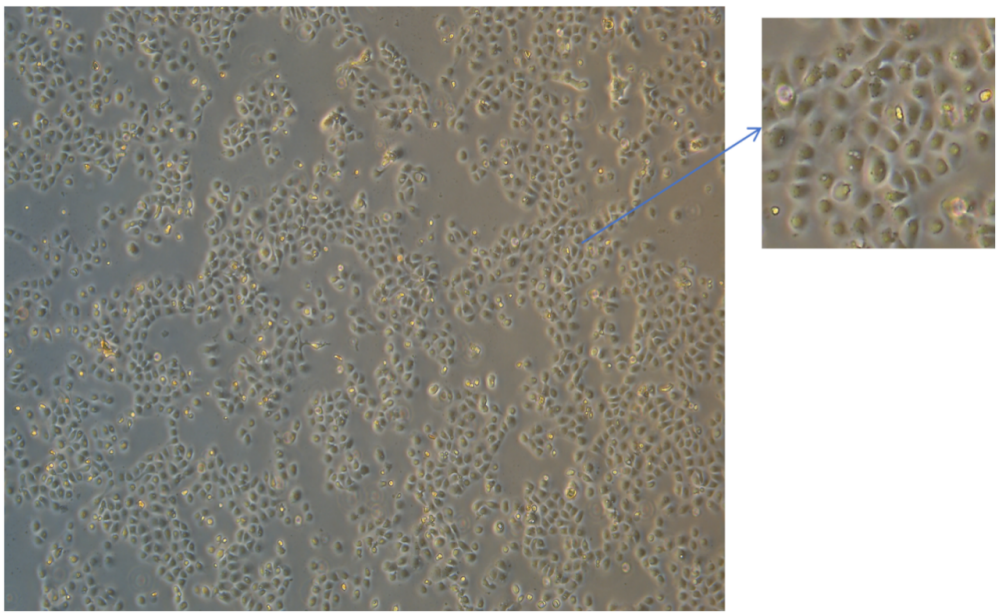


Figure 43. VK2/E6E7 cells grow adherent after passage.(P7)

Figure 43 shows the growth of VK2 cells after passage. The growth stage is cell attachment 7 days after passage. It can be clearly seen that the number of cells increases after passage and a very small number of cells die. On the other hand, most of the vaginal epithelial cells adhered to the flask and gradually stretched after 48 hours, and the confluency reached 80-90% after 7-8 days of culture. It has the characteristics of epithelial cells, with a polygonal shape, clear outline, and strong refractive index. The cells are laid out like paving stones and have the morphology of epithelial cells. It can be seen from the picture that the cell density has increased, the cell shape has changed from a round shape that has just started to adhere to the wall, spreading out tentacles and spreading out on the bottom of the flask. The epithelial cells are arranged in a mosaic, and the boundaries between cells are clear.

5.2.2 Cell viability - MTT Assay

MTT assay is a method for detecting cell viability analysis and proliferation, also known as MTT colorimetry. The origin of colorimetry is that succinate dehydrogenase exists in the mitochondria of living cells, which can undergo a reduction reaction with exogenous MTT reagent to obtain a reduced product - water-insoluble blue-purple

crystalline formazan. This blue-purple crystal will be deposited in living cells and can be observed. On the contrary, dead cells cannot undergo this reduction reaction to obtain blue-purple crystals. Therefore, this reaction of obtaining blue-purple crystalline products in living cells through reduction reaction can detect the number of cell stocks and proliferation after a certain period of cell culture. On the other hand, the number of blue-purple crystals is proportional to the number of cell survival. According to research, MTT assay is widely used in the detection of cell life activity, including cytotoxicity tests, drug screening and delivery. This test method has high sensitivity, is easy to detect cells, is easy to operate, and is relatively low in cost. Based on this background, this section will conduct cell growth tests on three different scaffolds to explore the survival of vaginal epithelial cells on different scaffolds, so as to explore the nanofiber membrane suitable for the growth of vaginal epithelial cells. In addition, the survival rate of cells on different scaffolds can also reflect the characteristics of materials suitable for the growth of vaginal epithelial cells, including the mechanical properties, biocompatibility and surface morphology of the materials.

The MTT assay experiment is divided into three groups of different scaffolds, namely chitosan/polyvinyl alcohol scaffolds, chitosan/polyvinyl alcohol oriented scaffolds and PCL/Gelatin scaffolds. It should be noted that the reason for adding PCL/Gelatin scaffolds in this part is to compare with previous studies, so as to explore the factors and conditions suitable for the growth of vaginal epithelial cells. On the other hand, the effects of different materials on the growth of vaginal epithelial cells were also explored. The parameters of the PCL/Gelatin scaffolds in this part refer to the optimal parameters used by Angel for comparison. The specific steps of the MTT assay experiment are as follows:

1. The three different scaffolds were placed in a vacuum drying oven for cross-linking treatment for 48 hours. After cross-linking treatment, the three different scaffolds were cut into circular films with a diameter of 1 cm. The three groups of scaffolds were set as three experimental groups, and a blank group was set as a control. Nine circularfilms were prepared for each of the three different scaffolds.

2. Pretreatment of the scaffold; The growth of vaginal epithelial cells on the scaffold requires thorough disinfection and sterilization to ensure the microenvironment for the cell growth on the scaffold. Firstly, the circular film needs to be peeled off from its carrier tin foil. As mentioned above, this process is a more difficult process. Electrospinning scaffolds have a certain viscoelasticity, which makes the film prone to wrinkles and the shape is not easy to complete. Therefore, with the help of liquid, including ethanol or pure water, the film peeling process can be smoother to ensure the integrity of the film shape. The peeled film is rinsed in ethanol three times for five minutes each time to ensure the disinfection of residual reagents after cross-linking. After ethanol rinsing, it needs to be rinsed with PBS liquid three times for five minutes each time to ensure the removal of residual ethanol liquid to avoid affecting the growth of cells on the scaffold. The washed scaffolds were stored in a 24-well plate containing a small amount of PBS for subsequent use.

3. Preparation of MTT solution. MTT reagents are all 100 mg of yellow powdered chemicals, so in the MTT assay, it needs to be prepared into an MTT solution that can be used in the experiment in advance, so as to facilitate the subsequent experimental operations. 100 mg of MTT powder needs to be prepared into a 5% concentration solution, that is, add 100 mg of powder to 20 ml of PBS solution and slowly stir to dissolve. The specific steps of this operation are to add 20 ml of PBS solution to a 50 ml centrifuge tube, use a pipette to draw 1 ml of PBS liquid into a small tube containing MTT powder, and repeatedly pipetting until the yellow powder is completely dissolved into a liquid. Then add it all at once to the centrifuge tube containing PBS liquid and mix evenly. It is necessary to sterilize the evenly mixed MTT solution. This is done by filter sterilization through a 0.22 micron filter. After sterilization, divide it into aliquots for storage. It should be noted that the MTT reagent is a photosensitive chemical reagent, and light protection must be performed during the preparation process and storage. For example, use tin foil or a light-proof bag to wrap the centrifuge tube containing the MTT solution to achieve light protection.

4. Cell counting operation. There are requirements for the number of cells implanted on the chitosan/polyvinyl alcohol scaffold. Too many cells will lead to insufficient space for cell growth, thereby inhibiting normal cell adhesion and differentiation, causing cell death. Too few cells will slow down cell growth, thereby prolonging the test cycle. Therefore, this section needs to explain the specific operation of cell counting.

10 microliters of cell suspension was added to the hemocytometer cell counting plate. When transferring the cell suspension to the cell counting plate, add it from the edge of the slide so that the suspension can completely fill the space of the counting plate. Next, let the counting plate stand for 2-3 minutes and observe whether there are bubbles inside it. The presence of bubbles will affect the observation results under the microscope. There is a mesh grid inside the cell counting plate, which clearly shows the distribution of cells in the grid. Figure 44 shows the counting process of the cells. The counting principle is: count up but not down, count left but not right. The judgment standard is whether it touches the middle line of the three side lines. For example, after selecting a 4x4 large grid, start counting from the small grid in the upper left corner. The cells at the edge of the grid are counted only once, and can be counted in order from left to right or from top to bottom. After the counting in the 4x4 large grid is completed, record the cell count value. Generally speaking, three different 4x4 large grids should be selected and counted three times, and the average of the three results is the cell counting value.

The statistical method of cell counting is to select the principle of single-side counting in the 4x4-grid (count the left but not the right, count the top but not the bottom), so as to count the number of cells in the 4x4-grid as a whole. Usually three 44-grid areas are selected, and the average of three cell counts is taken as the data for calculation.

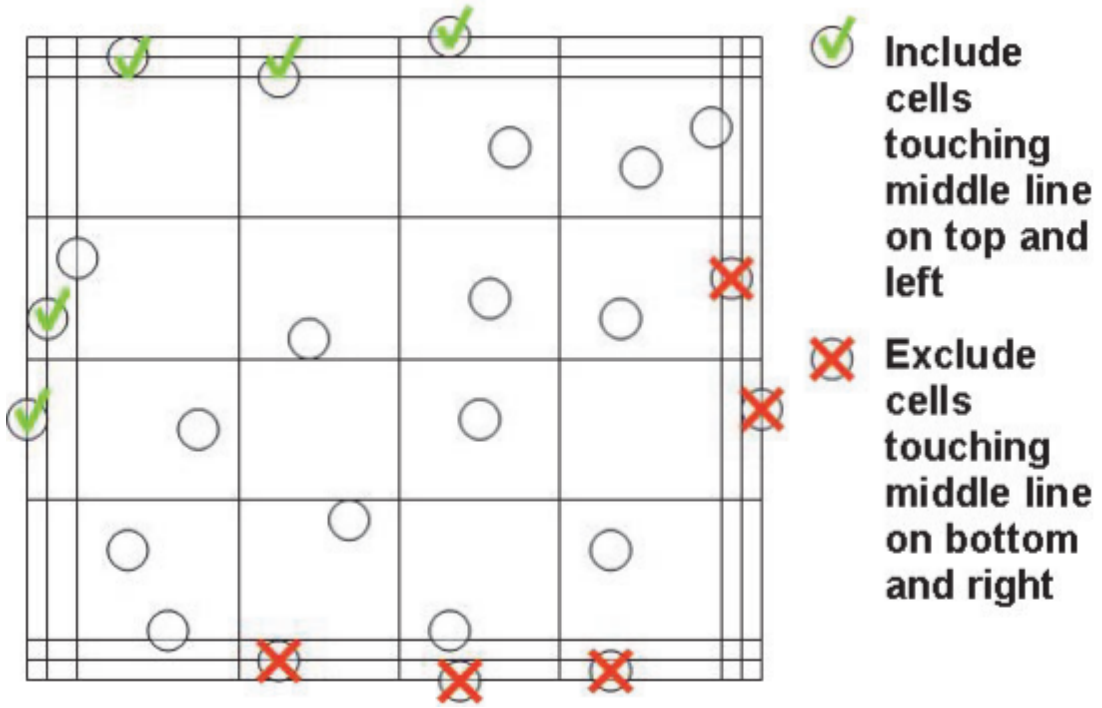


Figure 44. Cells counting principle

According to the cell concentration dilution formula $C_1V_1=C_2V_2$, the number of cells in the cell suspension obtained by counting is known, that is, the concentration C_1 of the original cell suspension; V_1 is the required cell Volume; C_2 is the desired cell concentration; V_2 is the Volume that needs to be prepared. Therefore, the required culture medium Volume is the difference between V_2 and V_1. Based on the above four items, they are all preparations before the MTT assay experiment, including the pretreatment of the scaffold, the preparation of the MTT solution and the counting of cells. The following steps are the specific operation steps in the MTT assay.

1. MTT assay operation process

The MTT test results were selected for the data of cells grown on three different scaffolds for three days, five days and seven days. Thus, three different 24-well plates are needed to culture cells on three different scaffolds. As shown in Figure 45, a 24-well plate culture diagram with three different scaffolds is placed, namely Cs/PVA 4%, Cs/PVA Oriented 4% and PCL/Gelatin. 500 microliters of cell suspension

with calculated cell concentration is added to each well, and then placed in the incubator for culture.

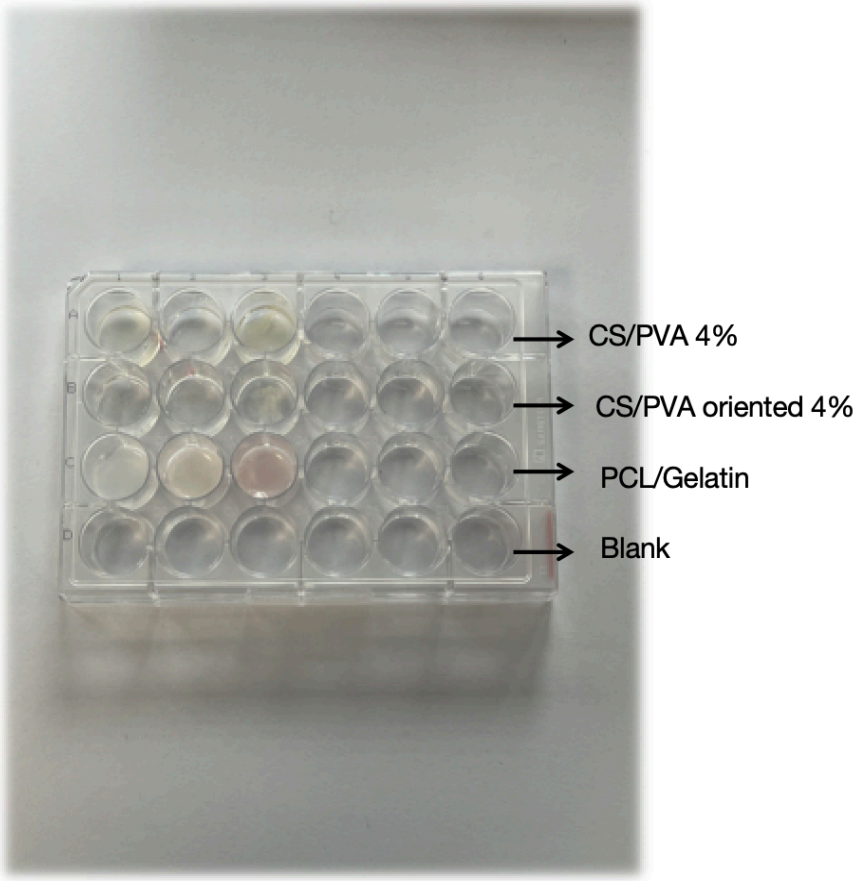


Figure 45. 24 well plate for three different scaffolds in MTT assay process

MTT assays were performed at 72h. Supernantant was removed from each well, take note not to remove the bottom scaffolds when sucking out the suspension. Then add 500 microliters of MTT solution to each well, cover the surface of the 24-well plate with tin foil or light-proof tape, and then sterilize the surface and place it in the incubator for 4 hours. After 4 hours, aspirate the MTT solution in each well and wash it with 500 microliters of PBS solution. This operation is to ensure that there is no residual free MTT solution in each well. After removing the PBS solution, add DMSO solution, 500 microliters per well, and then place it on a shaker to shake for 20 minutes. It should be stated that all operations must be protected from light. After shaking, it can be observed that the color of the solution in each well changes

significantly. The number of blue-purple crystals produced represents the depth of the solution color, and also indirectly confirms the number of cell stocks. Then transfer the three different solutions to a 96-well plate, 200 microliters per well, and read the MTT data. MTT data is often presented as absorbance values, which are directly proportional to cell survival. Figure 46 shows the calculation method of cell viaility in MTT Assay. In this experiment, the experimental group was cells cultured on three different scaffolds, the control group was cells cultured without scaffolds, and the blank control group was culture medium without cells..

$$\text{Cell viaility}(\%) = \frac{\text{absorbance value of the experimental group - absorbance value of the blank control}}{\text{absorbance value of the control group - absorbance value of the blank control}}$$

Figure 46. Calculation formula for cell viaility in MTT Assay

5.2.3 Live cell fluorescence imaging

Live cell imaging is a research method that studies the cellular structure and function of living cells through microscopic observation. It can display and quantify the dynamic changes of cells in real time and can study cellular and subcellular structure, function, and organization in living systems, helping to develop assays that are more biologically relevant and better predictive of the body response to new drug candidates. Live cell imaging can be used in a variety of research directions and biological applications, including long-term kinetic monitoring and fluorescent label detection of living cells.

The specific fluorescence operation as follows:

① After passage of the cells, seeded the cells into a 96-well plate and observed the adherent growth of the cells two days later.

② Added the compound to the cell suspension. The reaction period depended on the action mechanism of the compound and the biological reaction being studied. For example, apoptosis or receptor internalization assays typically require shorter incubation times, whereas multiparametric cytotoxicity assays require onger. If

compound processing takes longer, it may be necessary to replace compounds during incubation.

③ Used fluorophore staining dihydrochloride (DAPI). When staining, experimental rules should be followed. Live cell applications do not require staining. Label-free imaging and analysis can only track and monitor cells through brightfield imaging.

④ After adding the dye and compound, place the 96-well plate into the imaging instrument with integrated environmental control function, and obtain cell imaging according to the experimental market.

5.2.4 In vitro culture of vaginal chip based on Elveflow microfluidic system

As mentioned in the literature review section 2.5 above, the essence of in vitro culture of organ on a chip is the external regulation of microfluidic chips and flow rate control circulation systems, including the control of liquid flow rate, conversion between liquid channels and material circulation. The difficulty of in vitro culture lies in simulating the flow of liquids and material exchange in different tissues and organs in the human body, so as to simulate the real physiological microenvironment inside the human body. For example, in the construction of vaginal chips, the scaffold serves as a growth-bearing space for cells, which provides simulated organ tissue characteristics, and its superior mechanical properties and biocompatibility provide a tissue basis for in vitro culture. On the other hand, for in vitro culture, it is to build a sustainable cyclic liquid control system to simulate the fluid environment inside the vagina. Based on the above description of vagina on a chip in vitro culture, this part builts a platform integrating vagina on a chip and microfluidic control system to simulate the physiological microenvironment inside the vagina, so as to provide a better environment for the in vitro culture of vaginal epithelial cells. This part uses the microfluidic flow and pressure controller made by Elveflow to control the liquid flow rate and circulation system inside the vagina on a chip.

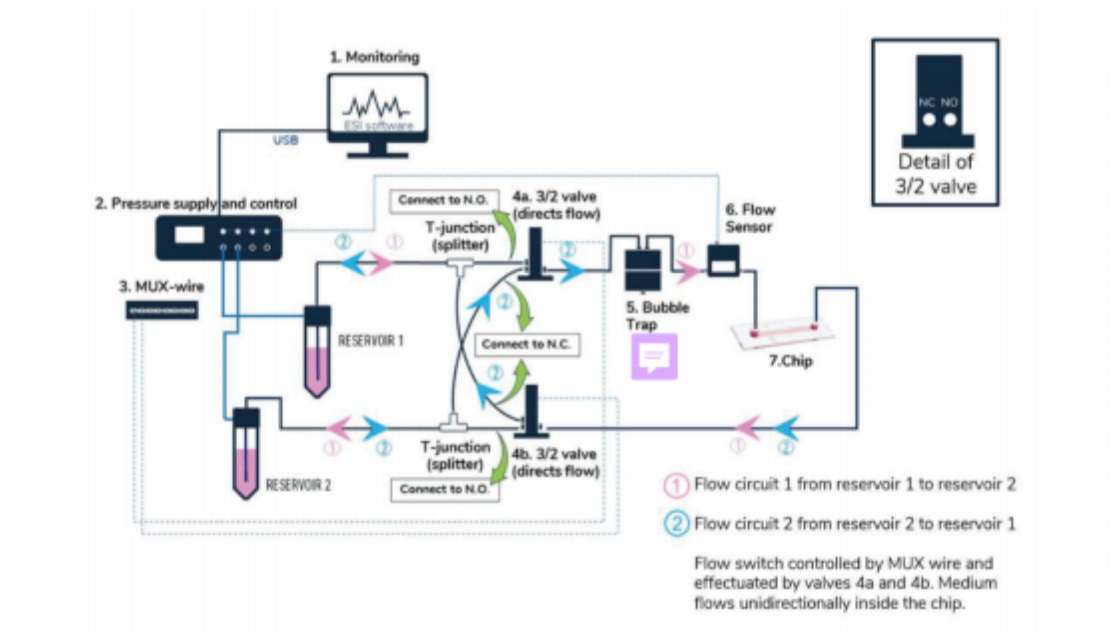


Figure 47. Elveflow equipment control schematic(https://www.elveflow.com/microfluidic-applications/microfluidic-cell-culture/medium-recirculation-using-microfluidic-valves/)

Figure 47 is a process diagram of the Elveflow device controlling the entire platform liquid flow and circulation system. Its control system is a complex process. Firstly, the corresponding pressure and flow rate of the liquid in the microfluidic chip to be controlled must be set on the computer software, so that the characteristics of liquid flow in the human body can be effectively simulated. For example, the culture medium and corresponding nutrients will be transmitted to the T junction through the pipeline under pressure control to achieve the change in the direction of the liquid flow rate. This process can effectively control the circulation of the liquid in the entire system, thereby ensuring the formation of a circulation system for the culture medium and waste liquid. Secondly, the bubble trap can effectively control the bubbles in the liquid in the pipeline through the pressure difference, thereby ensuring the smoothness of the liquid flow in the system, so that the nutrients can be smoothly transported to the chip, and the liquid control of cell culture in vitro can be realized.

In the actual construction of the in vitro culture platform, the preliminary operation can refer to the cell counting part of the MTT experiment operation in 5.2.2. After completing the preliminary operation, the counted cell suspension needs to be transferred to the integrated vagina on a chip. This process is a relatively difficult process. After trying different methods, the author finally determined the operation mode that minimizes cell damage and is effective. Firstly, putting the counted cell suspension into a T-75 flask for subsequent use, usually 10 ml of cell suspension. Then preparing a 10 ml syringe and a 0.5 mm diameter needle. Both the syringe and the needle need to be disinfected and sterilized. The pipette gradually draws the cell suspension in the T-75 flask into the 10 ml syringe. During this process, the syringe needs to be laid flat to prevent the cell suspension from leaking from the needle and causing contamination. Aligning the needle with the small tubes of the four connecting channels above the chip, and carefully push the cell suspension into the pipe. It is necessary to observe the flow direction of the liquid in the chip to prevent the liquid from flowing out from the opposite direction of the pipe. After the cell suspension passes through the chip support, the four channels above the chip are sealed, and the chip is sterilized and transferred to an incubator for 48 hours.

Figure 48. Integrated Elveflow control system and vagina on a chip

After 48 hours of culture, the chip was connected to the Elveflow system to achieve the overall construction of in vitro cultured vaginal epithelial cells. Figure 48 shows the actual process of integrating the Elveflow control system and the vagina on a chip, from which it can be clearly seen that the pipeline connection process is relatively complicated. Its purpose is to achieve the circulation of liquid in the pipeline and chip while controlling the liquid flow rate, so that the culture medium and waste liquid can be input and output in time.

5.3 Result Analysis

5.3.1 Cell Attachment Electrospun Membrane

Figure 49 mainly shows the electron microscope pictures of V2/E6E7 cells attached to the chitosan/polyvinyl alcohol electrospun membrane, it can be clearly seen that

the vaginal cells are organically combined with the fibers and adhered to the fibers. The cells were mainly cultured on the 24-well containing nanofibers for 7 days, then taken out and tested by electron microscope.

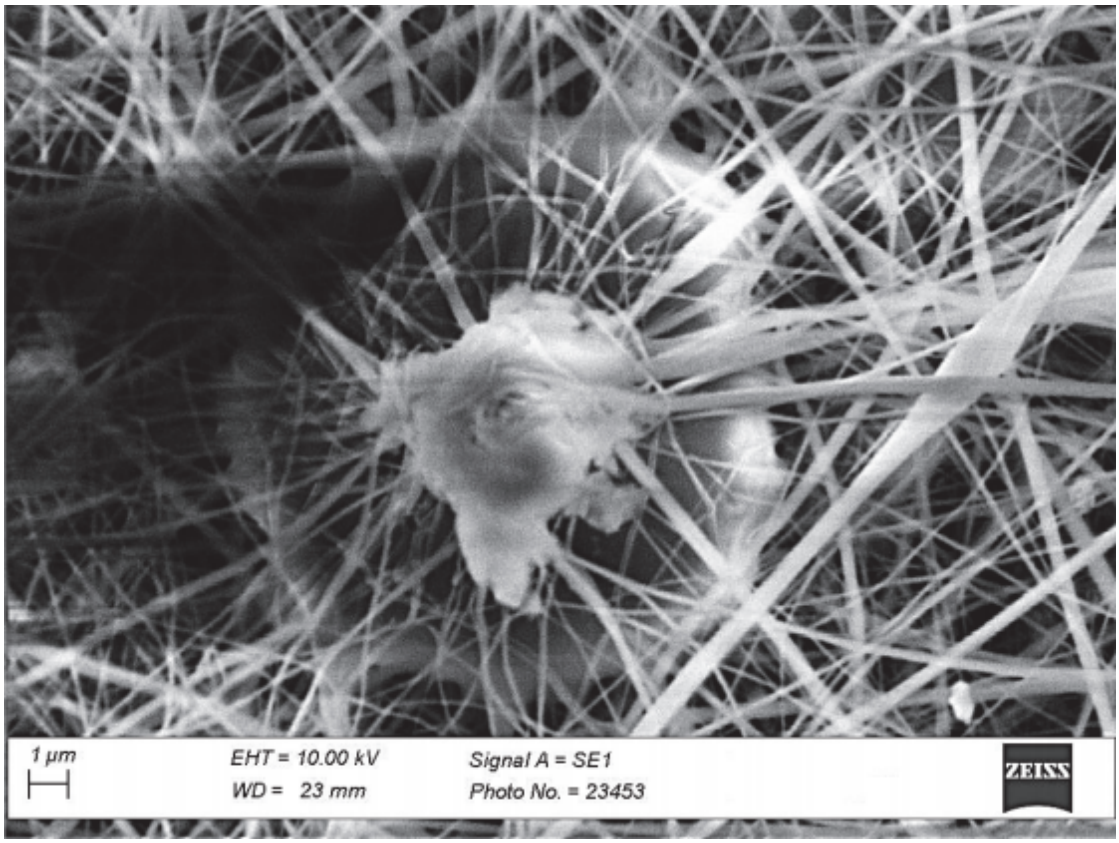


Figure 49. SEM image of VK2/E6E7 cell attach on the electrospun membrane

The results based on this SEM image confirmed that the chitosan/polyvinyl alcohol electrospun membrane has good cytocompatibility, and also verified that cells can grow and differentiate normally on the nanofibers. On the other hand, the morphology of cells adhering to the chitosan/polyvinyl alcohol scaffold shown in the shadow position in the middle also confirms the good biocompatibility of the chitosan material itself mentioned above. The results of cell adhesion also prove that the chitosan material itself has the effect of promoting cell adsorption. The confirmation of this result provides an experimental basis for the growth of vaginal epithelial cells on the vaginal chip, which also makes the vagina on a chip as a platform for vaginal cell growth and differentiation, which can more effectively study the pathological process of bacterial vaginosis, including the subsequent reaction and implantation of bacteria and cells on the platform. In addition, the SEM results show that the cell structure of the shadow part is intact and has a complete cell

morphology. This also shows that the growth of vaginal epithelial cells on the scaffold is not damaged, and the interaction between cells and scaffold is positive..

5.3.2 Cell survival and growth results

In each MTT experiment, three different groups of scaffolds described in section 5.2.2 were used in each MTT experiment. The purpose was to be able to average the data obtained during the MTT data analysis to make the experimental results more reliable. Thus, the analysis results of the MTT data were the average values of the cell survival rate data of the three electrospun membranes, and then analyzed. Figures 50 and 51 show the results of MTT data analysis at two different wavelengths, 570 nm and 630 nm. Data after cells grew on the scaffold for 3 days, 5 days and 7 days were selected to compare the cell culture results of three different scaffolds.

As showed in Figure 50, the cell survival rate at 570nm clearly shows that the chitosan/polyvinyl alcohol oriented scaffold has the highest cell inventory rate, which is the highest value at 3 days, 5 days and 7 days of cell growth. Secondly, the cell survival rate of the PCL/Gelatin scaffold is higher than that of the chitosan/polyvinyl alcohol scaffoldat 3 days and 5 days. However, after the cells grow to 7 days, the cell survival rate on the PCL/Gelatin is lower than that on the chitosan/polyvinyl alcohol scaffold. The reason why the cell inventory results of chitosan/polyvinyl alcohol scaffolds on the seventh day are better than those of PCL/Gelatin is that the performance of the material itself is different from the mechanical properties it provides. Thanks to the characteristics of chitosan own polyamino groups, it provides the scaffold with superior mechanical properties, making the scaffold have higher ductility and fracture strength; the hydrophilicity of polyvinyl alcohol polyhydroxyl groups improves the properties of chitosan to a certain extent, thereby increasing the degree of cell adsorption on the scaffold. On the other hand, the peristaltic muscle tissue inside the vagina determines that the growth microenvironment of vaginal epithelial cells requires stronger mechanical characteristics. As cells grow and differentiate on the scaffold, the cells occupy the space on the surface of the scaffold after they become more

numerous, which means that the scaffold itself needs to have mechanical properties similar to the peristalsis of muscle tissue, so that vaginal cells can survive longer. Although PCL itself also has excellent fracture strength and toughness, its biocompatibility is obviously not as good as chitosan, which is one of the reasons why the survival rate of cells on its surface decreases over time.

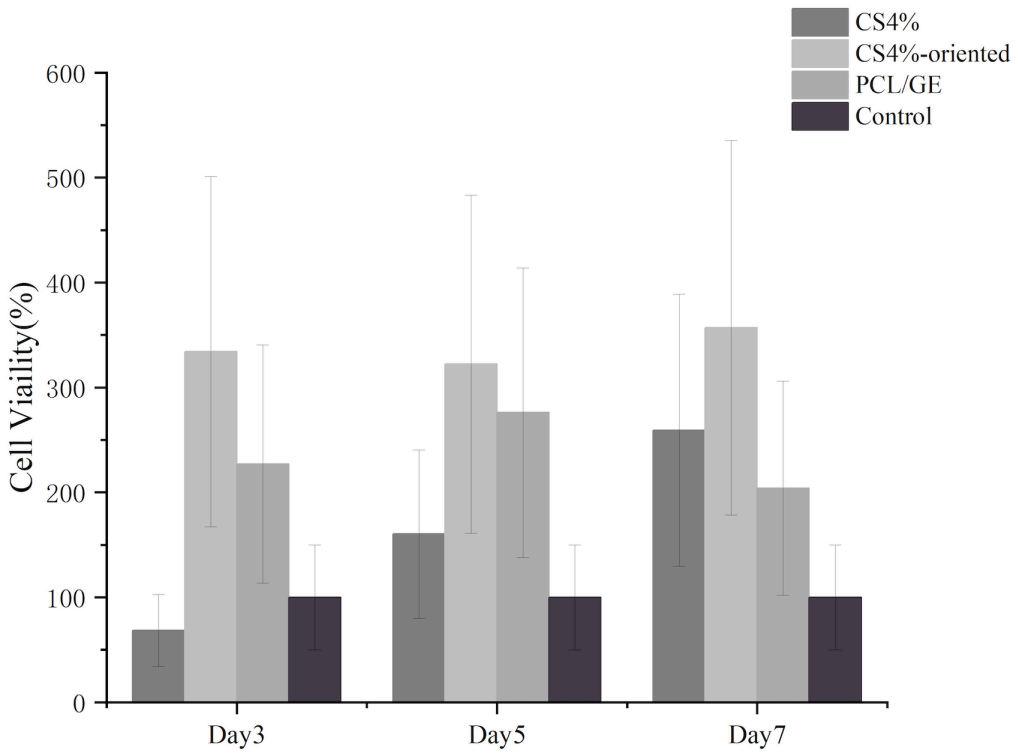


Figure 50. Cells growth Result of Cs/PVA and PCL/GE scaffolds (570nm)

From the comparison between the chitosan/polyvinyl alcohol scaffold and the oriented scaffold, it can be found that the survival rate of cells on the oriented scaffold is the best. This phenomenon confirms that the mechanical properties of the scaffold with increased orientation have a positive effect on the growth of vaginal epithelial cells. Secondly, the fiber diameter of the oriented scaffold is thinner, which means that there are more fibers in the same surface area. More fibers in the same area provide the scaffold with better toughness to ensure that the scaffold is not easily damaged by external factors. This also shows that the performance of the scaffold of oriented fiber electrospinning is superior.

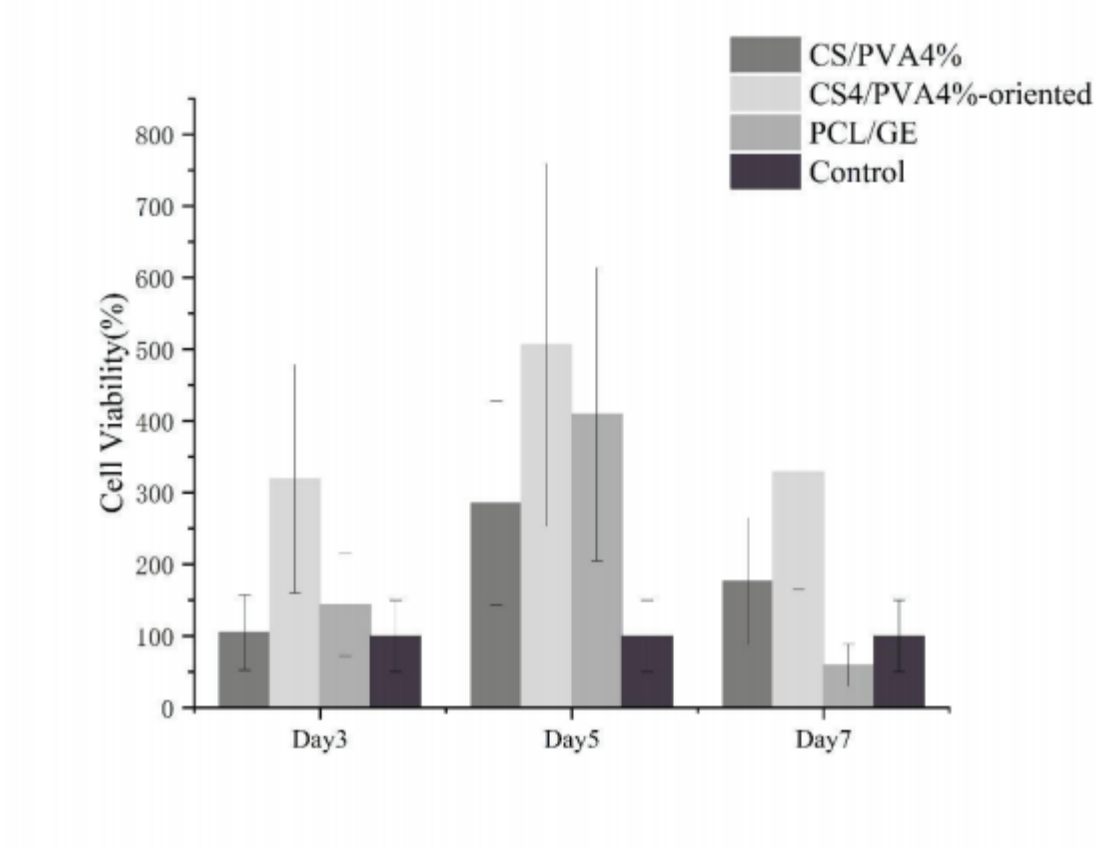


Figure 51. Cells growth Result of CS/PVA and PCL/GE Scaffolds (630nm)

The data results at 630nm shown in Figure 51 are consistent with the results at 570nm. The chitosan/polyvinyl alcohol oriented scaffold has the highest cell inventory rate, which is the highest value at 3 days, 5 days and 7 days of cell growth. The cell inventory rate on the PCL/Gelatin scaffold was lower than that of the other two scaffolds on the seventh day. The above description shows that the PCL material itself also has certain mechanical properties, but its strength is poor. The PCL functional group itself is a blend of 5 non-polar methylene-CH_2 starch and other substances, and its plasticity is good. However, the poor strength proves that it cannot provide the high-strength mechanical properties required for the growth of vaginal epithelial cells. The analysis results of the MTT data once again confirmed this conclusion.

5.3.3 Fluorescence tracking of cells on a vagina on a chip scaffold

Fluorescence tracking clearly demonstrated the cell growth structure and cell characteristics of three different scaffolds within the vagina on a chip, which

can effectively show the morphology and growth of vaginal epithelial cells cultured in vitro.

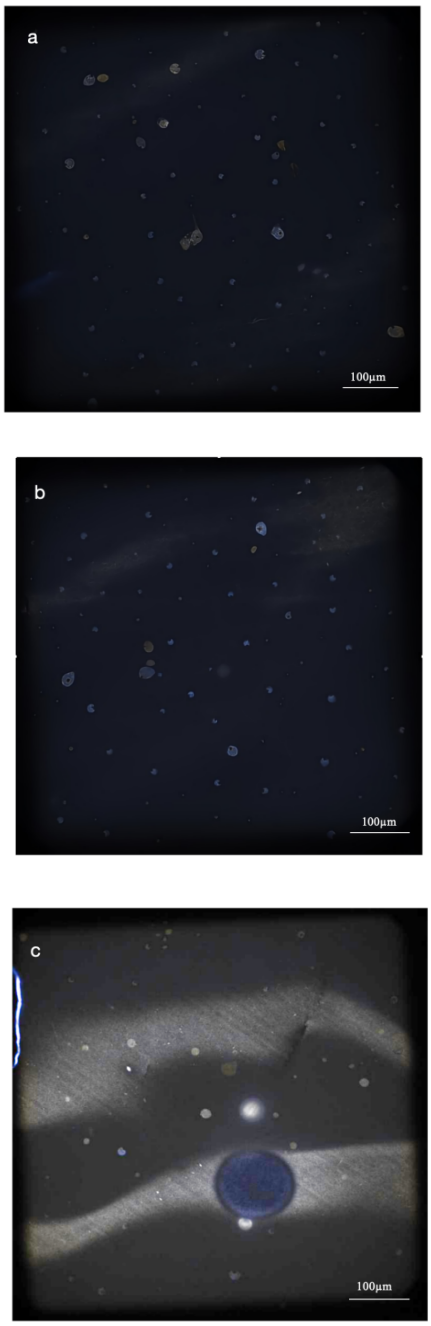


Figure 52. Fluorescence tracking of cells on the scaffolds within the chip. (a) Cs/PVA 4% scaffold;(b) Cs/PVA Oriented 4% scaffold and (c) PCL/Gelatin scaffold.

Figure 52 shows the results of fluorescence tracking under in vitro culture of three different scaffolds. First of all, in terms of cell number, the number of cells cultured in

vitro on the chitosan/polyvinyl alcohol oriented scaffold (b) is the largest, followed by the chitosan/polyvinyl alcohol scaffold (a), and the number of cells on the PCL/Gelatin scaffold (c) is the smallest. Secondly, from the perspective of cell structure, (b) can clearly show the edge characteristics of cells under fluorescence, showing elliptical and polygonal shapes, and the cell nucleus is obvious. The image of (c) shows that the small number of cells affects the imaging results, and the blue circle in the middle is caused by the bubbles on the PCL/Gelatin scaffold. This also shows that when the PCL/Gelatin scaffold is used to culture vaginal epithelial cells in vitro, the poor mechanical properties of the scaffold cause bubbles to appear due to the fluid shear force generated when the liquid passes through, thereby affecting the normal growth and differentiation of the cells. The cell structure characteristics of (a) are similar to those of (b), but its cell number is relatively small.

On the other hand, the results of the cell fluorescence test also confirmed that the mechanical properties of the scaffold are crucial to the growth of vaginal epithelial cells. The mechanical properties simulate the peristalsis of the muscle tissue inside the vagina, which promotes the growth of real human vaginal cells. The high ductility and fracture strength of the chitosan/polyvinyl alcohol scaffold can truly simulate the physiological microenvironment inside the vagina. The results of the fluorescence image also confirmed that its scaffold is conducive to cell proliferation, and the number is the largest among the three scaffolds. The adsorption characteristics of chitosan/polyvinyl alcohol for cells are also reflected in the fluorescence image.

6. Discussion

The above chapters give a detailed description of the construction mechanism and process of the vaginal chip platform. In terms of the overall structure, the vaginal chip platform constructed by this project is divided into three parts.

1. Select appropriate materials and tissue engineering technology to make a scaffold suitable for the growth of vaginal epithelial cells. In this part, the selection of materials and the application of electrospinning technology increase the feasibility of in vitro culture of vaginal epithelial cells. Chitosan is the product of N-deacetylation of chitin. There are a large number of free amino groups with positive charges in its molecular structure, which means that it can react chemically with negatively charged chemical bonds, thereby modifying the structural properties of the material. On the other hand, chitosan is easy for molecules to form electrostatic adsorption, which provides a theoretical basis for its application in the field of tissue engineering. The main purpose of selecting chitosan as the main biomaterial for the preparation of scaffolds in this project is to apply the excellent mechanical and biological properties of chitosan. The free amino groups provide chitosan materials with excellent strength and ductility, and the easy reaction of negatively charged molecules allows chitosan to combine with other materials to react in accordance with the reaction, thereby modifying the properties. Polyvinyl alcohol molecules contain many free hydroxyl groups, which makes it very easy to polymerize with cations in chitosan, thereby improving the solubility of chitosan.

From the perspective of electrospinning, the multi-aggregated cations in chitosan and the bonding effect of the solution have a serious hindering effect on electrospinning, which hinders the fiberization characteristics of the solution. This problem also leads to poor morphology of chitosan electrospinning-supported fibers, mostly bead-like fibers, and cannot form continuous and uniform fibers. The operation of electrospinning chitosan and polymer blends has also become a hot topic in research. For example, Li used polyethylene oxide and chitosan to blend and

spin chitosan to obtain chitosan/polyethylene oxide nanofibers; He obtained chitosan/polycaprolactone nanofibers by electrospinning a blend of chitosan and polycaprolactone. However, in order to promote the fiberization of chitosan, the chitosan content is low and the content of other polymers is high in the blended spinning of these polymers and chitosan, which is not conducive to studying the structural characteristics of the chitosan material itself. Based on the background, this study explored the properties of chitosan electrospun fibers by blending chitosan and polyvinyl alcohol in a certain proportion, and ensured the chitosan content in the blend, providing an experimental basis for studying the structural properties of chitosan materials.

2. The second main part of the project explored the design and fabrication of a chip suitable for the growth of vaginal epithelial cells, which included the modification of the chip size and the integration of the chitosan/polyvinyl alcohol scaffold and the chip. The organ on a chip section of the literature review elaborates on the principles of designing different organ chips, and it can be clearly seen that the design of the chip is based on the characteristics of human organs and tissues. For example, the gas exchange characteristics of organs, liquid flow and muscle tissue peristalsis. The purpose of simulating organ characteristics is to provide a physiological microenvironment that satisfies the growth of cells in vitro. For vaginal tissue, its biggest simulation characteristic is the muscle shear force provided by the peristaltic muscle tissue, thereby ensuring the mechanical properties of vaginal cell growth. The mechanical properties of this part are achieved through a scaffold prepared by electrospinning. The second is the complex fluid environment inside the vagina, including the flow and circulation exchange of liquids. This point is achieved through an externally controlled microfluidic system.

The main construction of the vagina on a chip platform is completed through the above two parts of the design characteristics of the scaffold chip, which provides a theoretical basis for the in vitro culture of vaginal epithelial cells. However, the

integration of chitosan/polyvinyl alcohol scaffolds and chips is a complex and difficult process. This process needs to ensure the integrity of the chip structure and the compatibility of the scaffold and chip, including ensuring that the scaffold morphology is not damaged, and the integration method does not affect the growth of cells and the ease of operation. Consequently, the author tried different methods to integrate the scaffold and chip, and explored the most effective way to integrate the two with the least damage to the chip and scaffold to construct a three-dimensional in vitro vaginal cell culture platform.

3. The third part of the project is the biological test of the vagina on a chip platform, which aims to explore the feasibility and effect of the platform in culturing vaginal cells in vitro. The comparison between different scaffolds was also explored to determine the scaffold that is most suitable for the growth of vaginal epithelial cells, and the influence of different material properties on the growth of vaginal cells was also confirmed from the side. For example, the cell culture method and cell survival rate test confirmed the effect of vaginal cells growing on different scaffolds, thereby analyzing the factors affecting cell growth; fluorescence tracking detected the structural characteristics of cells growing in the vaginal chip, providing an experimental theoretical basis for the subsequent implantation of bacteria.

6.1 Evaluation of electrospun chitosan/polyvinyl alcohol scaffolds

This project explored the electrospinning process of four different chitosan/polyvinyl alcohol concentration scaffolds, and analyzed the morphology, strain stress, composition and crystal structure of the electrospun scaffolds at different concentrations. On the other hand, the differences between chitosan/polyvinyl alcohol scaffolds made by oriented electrospinning and non-oriented scaffolds were explored, and their improved characteristics were summarized, thus confirming that oriented electrospinning has an improving effect on the preparation of scaffolds.

In terms of fiber morphology, chitosan/polyvinyl alcohol scaffolds at low concentrations (2%) mostly produce beaded fibers, with cross-fiber morphology and discontinuous and uneven fiber formation. This is because when the chitosan concentration is low, its polyvinyl alcohol increases its solubility, causing chitosan to dissolve into more solution forms in the form of a non-Newtonian fluid, stimulating the increase in liquid during the electrospinning process, so that the fibers mostly form beaded fibers. When the chitosan concentration is high (5%), although the beaded fibers are reduced, the increase in concentration leads to an increase in the viscosity of the solution, which increases the difficulty of the electrospinning process and is not conducive to experimental operation. Therefore, the chitosan/polyvinyl alcohol scaffolds at 3% and 4% concentrations have good morphology and continuous and uniform fibers. Subsequent mechanical property analysis will further screen them to determine the optimal parameters. On the other hand, the fiber morphology and diameter of oriented fibers and non-oriented fibers at a concentration of 4% were compared. It is clear that the oriented fibers have more uniform fiber arrangement and smaller fiber diameter, which confirms that the orientation degree of oriented electrospinning has an improving effect on the fiber morphology. The fiber diameter also indirectly shows that the number of oriented fibers in the same area of the scaffold is greater and its strength is also greater.

In the strain-stress analysis, as the chitosan concentration increases, its Young's modulus in the elastic stage is on an increasing trend; but when the chitosan concentration increases to 5 wt%, its Young's modulus in the elastic stage begins to decrease. This is because the increase in chitosan concentration increases the functional groups of the macromolecules in the solution, which cannot completely react with the negative ions in the polyvinyl alcohol, resulting in an unstable molecular structure in the solution. Based on the electron scanning microscopy morphology, fiber diameter distribution and strain-stress results, it is determined that the chitosan/polyvinyl alcohol scaffold at a concentration of 4% has the best

structural characteristics and effects. The oriented electrospinning effect at a chitosan concentration of 4% is the best.

The analysis of the components in chitosan/polyvinyl alcohol blends through infrared analysis explains the theoretical basis for the reaction after the two are combined. For example, in infrared analysis stretching vibration peaks of V(-OH) and V(-NH_2) of Cs at 3438 cm^{-1} broadened and moved toward lower wave numbers, indicating the formation of strong hydrogen bonds between Cs and PVA. Due to the effect of hydrogen bonding, the absorption peak of the functional group is shifted, and the characteristic absorption peak of δ (-NH2) at 1515 cm^{-1} also weakens. This is due to the addition of PVA, which reduces the proportion of amino groups. In addition, the characteristic absorption peaks of β-glycosidic bonds of chitosan at 1086 and 839 cm^{-1} almost disappeared, and the crystallization sensitive peak of chitosan at 664 cm^{-1} was also greatly weakened. This shows that the addition of PVA destroys the regularity of Cs molecular chains and reduces its crystallinity. The infrared spectra of the blend membranes of three different proportions are similar, and there is no new absorption peaks compared with the pure components, indicating that the blending of the two is only physical mixing.

X-ray diffraction analysis proved that if there is no interaction between Cs and PVA molecules in the electrospinning membrane, or the interaction is very weak, the diffraction peak of the membrane is a simple superposition of the crystallization peaks of the two components. However, it can be seen from Figure 33 that as the Cs content increases, the intensity of the crystallization peak of PVA at 2θ of 19.6° gradually weakens and broadens, which is consistent with reports in the literature. This further shows that there is a strong hydrogen bonding interaction between the two components in the Cs/PVA blend film. It is precisely because of this interaction that the original crystal structure of PVA is changed, thereby reducing the crystallinity.

6.2 Evaluation of chitosan/polyvinyl alcohol integrated vagina on a chip

The evaluation of the in vitro cultured vaginal cell platform integrating scaffolds and chips is mainly analyzed based on the survival rate of cells on it and the growth structure of cells. In addition, the difficulty, operation process and feasibility of the entire vagina on a chip construction are also evaluated. This has practical significance for the clinical application of vagina on a chip. Overly complicated operations and processes will increase the time cycle and cost of the experiment, while overly simplified processes cannot ensure the feasibility of in vitro culture of cells.

This project selected three different groups of scaffolds for electrospinning to make a comparison, so as to explore the most suitable conditions for the in vitro growth of vaginal epithelial cells. Chitosan/polyvinyl alcohol scaffold, oriented chitosan/polyvinyl alcohol scaffold and PCL/Gelain scaffold, according to the survival ratio of vaginal epithelial cells on the three scaffolds, reflected the influence of different material properties on cell growth. The MTT data results showed that the cell survival rate of oriented chitosan/polyvinyl alcohol scaffold was significantly higher than that of the other two scaffolds, and its survival ratio was higher. This once again confirmed the superiority of oriented electrospun fibers. The reason for the high survival rate is that the oriented scaffold has a more continuous and uniform fiber arrangement, which makes the scaffold have higher strength characteristics. A thinner fiber diameter means that there are more fiber roots in the scaffold of the same area, which increases the toughness and fracture strength of the scaffold. On the other hand, the result that the survival rate of the non-oriented scaffold on the seventh day was higher than that of the PCL/Gelatin scaffold proved that the good mechanical properties simulated the peristalsis of the muscle tissue inside the vagina and promoted the growth of vaginal epithelial cells. The results of fluorescence tracking showed the structural characteristics of cells in the chip after 15 days of growth. The cells in the three scaffolds all had round or polygonal structural

characteristics, and the cell nucleus were obvious. The number of cells in the oriented chitosan/polyvinyl alcohol scaffold was significantly higher than that in the other two scaffolds. On the other hand, the results of PCL/Gelatin fluorescence imaging also showed that there were many bubbles in its scaffold during in vitro culture, which would affect the normal growth of chip cells, making it impossible for the culture medium or nutrients to pass smoothly through the cross-channel in the chip.

In addition, the results of the successful in vitro culture of vaginal epithelial cells on three different scaffolds confirmed that the method of integrating scaffolds and chips in this project is feasible. While ensuring the smooth growth of cells, the complex operation process is simplified, and it can be effectively connected to external control, thereby realizing the control and circulation exchange of fluids in the chip.

7. Conclusion

7.1 Conclusion

This project used electrospinning technology to explore the spinning parameters of chitosan/polyvinyl alcohol blends and successfully spun a bioscaffold suitable for the growth of vaginal epithelial cells. At the same time, the improvement of oriented electrospinning on the in vitro culture of cells on the bioscaffold was further explored. The design of the improved vagina on a chip simplifies the complexity of the application of organ on a chip in clinical research, explores the most suitable method of integrating bioscaffolds and chips, and constructs a new vaginal chip platform, which provides experimental basis and theoretical basis for studying the pathological process of bacterial vaginosis. As a consequence, the main conclusions of this project are as follows:

1. The optimal parameters of the electrospun chitosan/polyvinyl alcohol scaffold were: 90 wt % acetic acid was used as solvent, the concentration of chitosan solution was 4 wt %; the spinning voltage was 23 KV and the receiving distance was 6.8 cm; the spinning flow rate is 0.8-1.2 ml per hour, and the humidity control needs to be adjusted according to the changes in the fiber during the spinning process; non-oriented fibers are typically 180±25 nm in diameter; the oriented chitosan nanofibers had an average diameter of 160±25 nm.

2. The strain stress value in the elastic phase of the electrospun scaffold determines the survival rate of vaginal epithelial cells cultured in vitro, including the number of cells and the cell growth structure. This conclusion was drawn from the comparison of oriented chitosan/polyvinyl alcohol scaffolds, non-oriented chitosan/polyvinyl alcohol scaffolds and PcL/Gelatin scaffolds. It was confirmed that in addition to the cell adsorption and sterility of the material, the ductility and fracture strength of the factors affecting the growth of vaginal epithelial cells have a promoting effect on the growth and differentiation of VK2/E6E7 cells.

3. A three-dimensional culture model suitable for the in vitro growth of vaginal epithelial cells was constructed, thereby integrating the vagina on a chip platform. The vagina on a chip can perfectly culture VK2/E6E7 cell lines in vitro, and confirms the promotion effect of chitosan/polyvinyl alcohol scaffold on VK2/E6E7 cell growth. It provides a feasible platform for clinical research on the pathological process of bacterial vaginosis and drug development and delivery.

7.2 Shortcomings and Future work

The project was launched in October 2020. Due to the spread and impact of the COVID-19 pandemic, the laboratories in the school were closed. The formal experimental part was implemented after the lock down in June 2021, which had a huge impact on the progress of the project. Therefore, the subsequent bacterial implantation experiment of the project was not implemented and the time schedule was not implemented to explore the reaction process of implanting relevant bacteria on the vagina on a chip and the VK2/E6E7 cell line.

1. This project has a relatively simple method for testing the mechanical properties of biological scaffolds. Limited by the accuracy of current commercial tensile instruments and the proficiency of operators, the tensile specimens of chitosan/polyvinyl alcohol nanofibers in this article are not fine enough and the number of samples is relatively small. Subsequent research should consider using diversified nanofiber stress tests to measure the mechanical properties of nanofibers, providing strong support for the construction of nanofiber membranes for tissue engineering scaffolds.

2. The pathological process of bacterial vaginosis is mostly caused by the overgrowth of anaerobic bacteria, including Gardnerella, Prevotella, Mobiluncus and bacterial vaginosis-related bacteria, which causes the disorder of vaginal microecological flora. This project did not implant different pathogens and the VK2/E6E7 cell line reaction

on the vagina on a chip to explore the immune response of pathogens to cells in vitro. In future operations, it is planned to implant different bacteria in vitro, including Lactobacillus and Gardnerella, to study the effects on vaginal cell pH and pro-inflammatory cytokines, so as to provide a more reliable theoretical basis for clinical research and drug development and delivery of vagina on a chip.

8. References

Anukam,K. & osazuwa, E. (2006). Augmentation of antimicrobial metronidazole therapy of bacterial vaginosis with oral probiotic Lactobacillus rhamnosus GR-1 and Lactobacillus reuteri RC-14: randomized, double-blind, placebo controlled trial. Microbes and infection, 8(6), p.1450-1454.

Augustine, R., Rehman, S. R. U., Ahmed, R., zahid, A. A., Sharifi, M., Falahati, M., & Hasan, A. (2020). Electrospun chitosan membranes containing bioactive and therapeutic agents for enhanced wound healing. International journal of biological macromolecules, 156, p.153-170.

Abbasi, N., Hamlet, S., Love, R. M., & Nguyen, N. T. (2020). Porous scaffolds for bone regeneration. Journal of science: advanced materials and devices, 5(1), p.1-9.

Ahmed, F. E., Lalia, B. S., & Hashaikeh, R. (2015). A review on electrospinning for membrane fabrication: Challenges and applications. Desalination, 356, p.15-30.

Angammana, C. J., & Jayaram, S. H. (2016). Fundamentals of electrospinning and processing technologies. Particulate Science and Technology, 34(1), p.72-82.

Agarwal, S., Greiner, A., & wendorff, J. H. (2013). Functional materials by electrospinning of polymers. Progress in Polymer Science, 38(6), p.963-991.

Abbe, C., & Mitchell, C. M. (2023). Bacterial vaginosis: a review of approaches to treatment and prevention. Frontiers in reproductive health, 5, 1100029.

Baji, A., Mai, Y. w., wong, S. C., Abtahi, M., & Chen, P. (2010). Electrospinning of polymer nanofibers: Effects on oriented morphology, structures and tensile properties. Composites science and technology, 70(5), p.703-718.

Bautista, C. (2016). Bacterial VA Gino sis: A synthesis of the literature on etiology, prevalence, risk factors, and relationship with Amyotrophic acid and gonorrhea infections. Military Medical Research, 3(4).

Beaurivage, C. et al. (2019). Development of a Gut-on-A-Chip Model for High Throughput Disease Modeling and Drug Discovery, Int J Mol Sci. 20 (22). doi: 10.3390/ijms20225661.

Bogorad, M. I., DeStefano, J., wong, A. D., & Searson, P. C. (2017). Tissue-engineered 3D microvessel and capillary network models for the study of vascular phenomena. Microcirculation, 24(5), e12360.

Biranje, S., Madiwale, P., & Adivarekar, R. V. (2017). Electrospinning of chitosan/PVA nanofibrous membrane at ultralow solvent concentration. Journal of Polymer Research, 24, p.1-10.

Babar, A. A., Iqbal, N., wang, X., Yu, J., & Ding, B. (2019). Introduction and historical overview. In Electrospinning: Nanofabrication and Applications (pp. 3-20). william Andrew Publishing.

Barua, R., Biswas, N., & Das, D. (2024). Emergent Applications of organ-on-a-chip (ooAc) Technologies with Artificial Vascular Networks in the 21st century. In Emerging Technologies for Health Literacy and Medical Practice (pp. 198-219). IGI Global.

Boyang Z, Anastaisa K, et al. (2018)IAdvances in organ-on-a-chip engineeringl, Nature Reviews Materials, 3, p.257-278.

Bradshaw, c. (2005). Evaluation of a Point-of-care Test, BVBlue, and clinical and Laboratory criteria for Diagnosis of Bacterial Vaginosis. Journal of clinical Microbiology,43(3).

Bradshaw, c., & Sobel, J. (2016). current Treatment of Bacterial Vaginosis—Limitations and Need for Innovation. The Journal of Infectious Diseases. 214(1), p.14-20.

catriona S., & Jack D. (2014). current Treatment of Bacterial Vaginosis—Limitations and Need for Innovation. The Journal of Infectious Diseases, 214(1), p.14-20.

coudray, M. & Madhivanan, P. (2020). Bacterial vaginosis—A brief synopsis of the literature. European Journal of obstetrics & Gynecology and Reproductive Biology. 245, p.143-148.carvalho, V., GonGalves, I., Lage, T., Rodrigues, R. o., Minas, G., Teixeira, S. F., ... & Lima, R. A. (2021). 3D printing techniques and their applications to organ-on-a-chip platforms: a systematic review. Sensors, 21(9),p. 3304.

caplin J. et, al. (2015). Microfluidic organ-on-a-chip Technology for Advancement of Drug Development and Toxicology. Advanced Healthcare Materials. 4(10).p. 1426-1450.

Cho, S., Lee, S., & Ahn, S. I. (2023). Design and engineering of organ-on-a-chip. Biomedical engineering letters, 13(2), 97-109.

clapp, N., & Amour, A. (2021). organ-on-chip application sin drug discovery: press end user perspective. Biochemical society transactions. 49(4). Do i:10.1042/B St20210840chen, M. c., Sun, Y. c., & chen, Y. H. (2013). Electrically conductive nanofibers with highly oriented structures and their potential application in skeletal muscle tissue engineering. Acta biomaterialia, 9(3), p.5562-5572.

chliara MA, Elezoglou S, Zergioti I. Bioprinting on organ-on-chip: Development and Applications. Biosensors (Basel). (2022) Dec 6;12(12):1135. doi: 10.3390/bios12121135. PMID: 36551101;

PMcID: PMc9775862 .chen, X., Xiao, z., cai, Y., Huang, L., & chen, c. (2022). Hypothalamic mechanisms of obesity-associated disturbance of hypothalamic-pituitary-ovarian axis. Trends in endocrinology & metabolism, 33(3), p.206-217.

cui, c., Sun, S., Wu, S., chen, S., Ma, J., & zhou, F. (2021). Electrospun chitosan nanofibers for wound healing application. Engineered Regeneration, 2, p.82-90.

cima, L. G., vacanti, J. P., vacanti, c., Ingber, D., Mooney, D., & Langer, R. (1991). Tissue engineering by cell transplantation using degradable polymer substrates.

Doryab, A. & Tas, S. (2019). evolution of bio engineered lung models: recent advance sand challenge sin tissue mimicry for studying the role of mechanical force since LL biology. Advanced functional materials, 29(39), p.103-114.

De Filippo, R. E., Yoo, J. J., & Atala, A. (2003). Engineering of vaginal tissue in vivo. Tissue engineering, 9(2), p.301-306.

De vrieze, S., Westbroek, P., van camp, T., & van Langenhove, L. (2007). Electrospinning of chitosan nanofibrous structures: feasibility study. Journal of Materials Science, 42, p.8029-8034.

Dong-dong, L. I., Qin-xia, P. A. N. G., Min, z. H. U., & Le, K. A. N. G. (2016). Progress on Methods

for Establishing Animal Model of vaginosis. Laboratory Animal and comparative Medicine, 36(4), p.317.

Dhandayuthapani, B., Yoshida, Y., Maekawa, T., & Kumar, D. S. (2011). Polymeric scaffolds in tissue engineering application: a review. International journal of polymer science, 2011(1), 290602.

Desai, K., Kit, K., Li, J., & zivanovic, S. (2008). Morphological and surface properties of electrospun chitosan nanofibers. Biomacromolecules, 9(3), p.1000-1006.

Deng, J., Wei, W., chen, z., Lin, B., zhao, W., Luo, Y., & zhang, X. (2019). Engineered liver-on-a-chip platform to mimic liver functions and its biomedical applications: A review. Micromachines, 10(10), p.676.

Doerflinger, S. Y., Throop, A. L., & Herbst-Kralovetz, M. M. (2014). Bacteria in the vaginal microbiome alter the innate immune response and barrier properties of the human vaginal epithelia in a species-specific manner. The Journal of infectious diseases, 209(12), p.1989-1999.

Dobrzahski, L. A. (2014). overview and general ideas of the development of constructions, materials, technologies and clinical applications of scaffolds engineering for regenerative medicine. Archives of Materials Science and Engineering, 69(2), p.53-80.

Eltom, A., Zhong, G., & Muhammad, A. (2019). scaffold techniques and designs in tissue engineering functions and purposes: a review. Advances in materials science and engineering, 2019(1), 3429527.

Echeverria Molina, M. I., Malollari, K. G., & Komvopoulos, K. (2021). Design challenges in polymeric scaffolds for tissue engineering. Frontiers in Bioengineering and Biotechnology, 9, 617141.

Eduok, U., Faye, O., & szpunar, J. (2017). Recent developments and applications of protective silicone coatings: A review of PDMs functional materials. Progress in Organic Coatings, 111, p.124-163.

Ertl, P., sticker, D., Charwat, V., Kasper, C., & Lepperdinger, G. (2014). Lab-on-a-chip technologies for stem cell analysis. Trends in biotechnology, 32(5), p.245-253.

Edwards, V. L., McComb, E., Gleghorn, J. P., Forney, L., Bavoil, P. M., & Ravel, J. (2022). Three-dimensional models of the cervicovaginal epithelia to study host-microbiome interactions and sexually transmitted infections. Pathogens and disease, 80(1), ftac026.

Fan, C., Basharat, Z., Mah, K., & wei, C. R. (2024). Computational approach for drug discovery against Gardnerella vaginalis in quest for safer and effective treatments for bacterial vaginosis. scientific Reports, 14(1), 17437.

Fichorova, R. N., Desai, P. J., Gibson III, F. C., & Genco, C. A. (2001). Distinct proinflammatory host responses to Neisseria gonorrhoeae infection in immortalized human cervical and vaginal epithelial cells. Infection and immunity, 69(9), p.5840-5848.

Fuller, R. (Ed.). (2012). Probiotics: the scientific basis.

Fu, N., Dong, T., Meng, A., Meng, Z., Zhu, B., & Lin, Y. (2018). Research progress of the types and preparation techniques of scaffold materials in cartilage tissue engineering. Current stem Cell Research & Therapy, 13(7), p.583-590.

Fuchs, s., Johansson, s., Tjell, A. ϕ., werr, G., Mayr, T., & Tenje, M. (2021). In-line analysis of organ-on-chip systems with sensors: Integration, fabrication, challenges, and potential. ACs Biomaterials science & Engineering, 7(7), 2926-2948.

R N Fichorova, J G Rheinwald, D J Anderson. (1997). Generation of papillomavirus-immortalized cell lines from normal human ectocervical, endocervical, and vaginal epithelium that maintain expression of tissue-specific differentiation proteins. Biology of Reproduction,57(4),p. 847

GA MaLF. M. & Mohammed s.(2014). Evaluation of different diagnostic methods of bacterial vaginosis. Journal of Dental and Medical sciences, 13(1), p.15-23.

Gilbert, N. M., Lewis, W. G., Li, G., sojka, D. K., Lubin, J. B., & Lewis, A. L. (2019). Gardnerella vaginalis and prevotella bivia trigger distinct and overlapping phenotypes in a mouse model of bacterial vaginosis. The journal of infectious diseases, 220(7), p.1099-1108.

Gnecco, J. s., Ding, T., smith, c., Lu, J., Bruner-Tran, K. L., & osteen, K. G. (2019). Hemodynamic forces enhance decidualization via endothelial-derived prostaglandin E2 and prostacyclin in a microfluidic model of the human endometrium. Human reproduction, 34(4), p.702-714.

Gao, W., Weng, J., Gao, Y., & chen, X. (2013). comparison of the vaginal microbiota diversity of women with and without human papillomavirus infection: a cross-sectional study. BMc infectious diseases, 13, p.1-10.

Gopinathan, J., & Noh, I. (2018). Recent trends in bioinks for 3D printing. Biomaterials research, 22(1), 11.

Heinrich, M. (2022). Impact of endotoxins on bioengineered tissues and models. Trends in Biotechnology, 40(5). p.532-534.

Huang. M. (2023). Assessing vaginal wall indexes in premenopausal versus postmenopausal women by transrectal linear array high-frequency probe, Eur J Med Res. 28(390).

Horzum, N., Mete, D., Karaku§, E., UGinci, M., Emrullaholu, M., & Demir, M. M. (2016). Rhodamine-immobilised electrospun chitosan nanofibrous material as a fluorescence turn-on Hg2+ sensor. chemistryselect, 1(5), p.896-900.

Ho, c. T., Lin, R. Z., chen, R. J., chin, c. K., Gong, s. E., chang, H. Y., ... & Liu, c. H. (2013). Liver-cell patterning lab chip: mimicking the morphology of liver lobule tissue. Lab on a chip, 13(18), p.3578-3587.

Huh, D., Matthews, B. D., Mammoto, A., Montoya-Zavala, M., Hsin, H. Y., & Ingber, D. E. (2010). Reconstituting organ-level lung functions on a chip. science, 328(5986), 1662-1668.

Jenifer, A. (2007). prevalence of bacterial VA Gino sis 2001-2004 national health and nutrition examination survey data. obstetrics & gynecology, 109(1),p.114-120.

Jackson, R., Maarsingh, J. D., Herbst-Kralovetz, M. M., & Van Doorslaer, K. (2020). 3D oral and cervical Tissue Models for studying papillomavirus Host-pathogen Interactions. current protocols in microbiology, 59(1), e129.

Jang, K. J., otieno, M. A., Ronxhi,J., Lim, H. K., Ewart, L., Kodella, K. R., ... & Hamilton, G. A. (2019). Reproducing human and cross-species drug toxicities using a Liver-chip. science translational

medicine, 11(517), eaax5516.

Jalili-Firoozinezhad, S., Gazzaniga, F. S., calamari, E. L., camacho, D. M., Fadel, c. W., Bein, A., ... & Ingber, D. E. (2019). A complex human gut microbiome cultured in an anaerobic intestine-on-a-chip. Nature biomedical engineering, 3(7), p.520-531.

Jack, D. & Sobel, MD. (2006). Suppressive antibacterial therapy with 0.75% metronidazole vaginal gel to prevent recurrent bacterial vaginosis. American Journal of obstetrics and Gynecology, 194(5), p.1283-1289.

Ji, D., Lin, Y., Guo, X., Ramasubramanian, B., Wang, R., Radacsi, N., ... & Ramakrishna, S. (2024). Electrospinning of nanofibres. Nature Reviews Methods primers, 4(1), 1.

kang, Y. B., Sodunke, T. R., cirillo, J., Bouchard, M. J., & Noh, H. (2013, June). Liver on a chip: Engineering the liver sinusoid. In 2013 Transducers & Eurosensors XXVII: The 17th International conference on Solid-State Sensors, Actuators and Microsystems (TRANSDUcERS & EURoSENSoRS XXVII) (pp. 301-304). IEEE.

kenyon, c,. colebunders, M. & crucitti, T. (2013). The global epidemiology of bacterial vaginosis: a systematic review Am J obstet Gynecol, 209(6), p. 505-523.

kim, S. S., Gwak, S. J., & kim, B. S. (2008). orthotopic bone formation by implantation of apatite-coated poly (lactide-co-glycolide)/hydroxyapatite composite particulates and bone morphogenetic protein-2. Journal of Biomedical Materials Research part A: An official Journal of The Society for Biomaterials, The Japanese Society for Biomaterials, and The Australian Society for Biomaterials and the korean Society for Biomaterials, 87(1), p. 245-253.

kostadinova, R., Boess, F., Applegate, D., Suter, L., Weiser, T., Singer, T., ... & Roth, A. (2013). A long-term three dimensional liver co-culture system for improved prediction of clinically relevant drug-induced hepatotoxicity. Toxicology and applied pharmacology, 268(1), p.1-16.

kwak, J., pandey, S., cho, J., Song, M., kim, E. S., Doo, H., ... & kim, H. B. (2023). Development of the standard mouse model for human bacterial vaginosis induced by Gardnerella vaginalis. Frontiers in Veterinary Science, 10, 1226859.

kim, T. Y., choi, J. W., park, k., kim, S., kim, J. F., park, T. E., & Seo, J. (2024). Lubricant-coated organ-on-a-chip for Enhanced precision in preclinical Drug Testing. Small, 2402431.

taniewski, p., Gomez, A., Hire, G., So, M., & Herbst-kralovetz, M. M. (2017). Human three-dimensional endometrial epithelial cell model to study host interactions with vaginal bacteria and Neisseria gonorrhoeae. Infection and immunity, 85(3), p.10-1128.

taniewski, P., & Herbst-kralovetz, M. M. (2021). Bacterial vaginosis and health-associated bacteria modulate the immunometabolic landscape in 3D model of human cervix. npj Biofilms and Microbiomes, 7(1), p.88.

Li, Y., zhu, J., Cheng, H., Li, G., Cho, H., Jiang, M., ... & zhang, X. (2021). Developments of advanced electrospinning techniques: A critical review. Advanced Materials Technologies, 6(11), 2100410.

Lu, L., zheng, J., & Mishra, s. (2014, october). A model-based layer-to-layer control algorithm for ink-jet 3d printing. In Dynamic systems and control conference (Vol. 46193, p. V002T35A001). American society of Mechanical Engineers.

taniewski, P., & Herbst-kralovetz, M. M. (2019). Analysis of host responses to Neisseria gonorrhoeae using a human three-dimensional endometrial epithelial cell model. Neisseria gonorrhoeae: Methods and Protocols, p.347-361.

Langer, R. (1995). Biomaterials and biomedical engineering. Chemical Engineering science, 50(24), p.4109-4121.

Li, z., wang, C., Li, z., & wang, C. (2013). Effects of working parameters on electrospinning. one-dimensional nanostructures: Electrospinning technique and unique nanofibers, p.15-28.

Leung, C. M., De Haan, P., Ronaldson-Bouchard, k., kim, G. A., ko, J., Rho, H. s., ... & Toh, Y. C. (2022). A guide to the organ-on-a-chip. Nature Reviews Methods Primers, 2(1), p.33.

Lorenzo-Martin, L. F., Hibscher, T., Bowler, A. D., Broguiere, N., Langer,J., Tillard, L., ... & Lutolf, M. P. (2024). spatiotemporally resolved colorectal oncogenesis in mini-colons ex vivo. Nature, p.1-8.

Li, Y., zhang, Q. Y., sun, B. F., Ma, Y., zhang, Y., wang, M., ... & zhu, L. (2021). single-cell transcriptome profiling of the vaginal wall in women with severe anterior vaginal prolapse. Nature Communications, 12(1), p.87.

Liu, X., & Ma, P. X. (2009). Phase separation, pore structure, and properties of nanofibrous gelatin scaffolds. Biomaterials, 30(25), p.4094-4103.

Mahajan, G. & Doherty, T. (2022). Vaginal micro biome-host interactions modeled in human vagina-on-the-ground. Micro Biome 10(201).

Martins, B. (2023). Bacterial vaginosis and cervical human papillomavirus infection in young and adult women: a systematic review and meta-analysis. Rev saude Publica, 56(113), p.1-8

Mohammadzadeh, F.(2015). Diagnostic value of Amsel,s Clinical Criteria for Diagnosis of Bacterial vaginosis. Glob J Health Sci, 7(3), p.8-14.

Ma, C., peng, Y., Li, H., & Chen, W. (2021). organ-on-a-chip: a new paradigm for drug development. Trends in pharmacological sciences, 42(2), p.119-133.

Myer, L. et al. (2005). Bacterial vA gin oSI sand susceptibility to HIv infection in south African women: A nested case-control study. The journal of infectious diseases, 192(8), p.1372-1380.

Nugent, R. & Krohn, M. (1991). Reliability of diagnosing bacterial vaginosis is improved by a standardized method of gram stain interpretation. Journal of Clinical Microbiology, 29(2).

Noh, H. K., Lee, S. W., Kim, J. M., oh, J. E., Kim, K. H., Chung, C. p., ... & Min, B. M. (2006). Electrospinning of chitin nanofibers: degradation behavior and cellular response to normal human keratinocytes and fibroblasts. Biomaterials, 27(21), p.3934-3944

oduyebo, o. (2009). The effects of antimicrobial therapy on bacterial vaginosis in non-pregnant women. Cochrane Database of Systematic Reviews, 3(19).

ohkawa, K., Cha, D., Kim, H., Nishida, A., & Yamamoto, H. (2004). Electrospinning of chitosan. Macromolecular rapid communications, 25(18), p.1600-1605.

pennella, F., Cerino, G., Massai, D., Gallo, D., Falvo D,Urso Labate, G., Schiavi, A., ... & Morbiducci, U. (2013). A survey of methods for the evaluation of tissue engineering scaffold permeability. Annals of Biomedical Engineering, 41, p.2027-2041.

park, J. S. (2010). Electrospinning and its applications. Advances in Natural Sciences: Nanoscience and Nanotechnology, 1(4), 043002.

phipps, M. C., Clem, W. C., Catledge, S. A., Xu, Y., Hennessy, K. M., Thomas, v., ... & Bellis, S. L. (2011). Mesenchymal stem cell responses to bone-mimetic electrospun matrices composed of polycaprolactone, collagen I and nanoparticulate hydroxyapatite. ploS one, 6(2), e16813.

pranzo, D., Larizza, p., Filippini, D., & percoco, G. (2018). Extrusion-based 3D printing of microfluidic devices for chemical and biomedical applications: A topical review. Micromachines, 9(8), p.374.

villaseca, R. & ovalle, A et al,.(2015). vaginal infection Sina family health clinic int and metropolitan region, Chile. Europe pMC, 32(1), p.30-36.

Raya-Rivera, A. M., Esquiliano, D., Fierro-pastrana, R., L6pez-Bayghen, E., valencia, p., ordorica-Flores, R., ... & Atala, A. (2014). Tissue-engineered autologous vaginal organs in patients: a pilot cohort study. The Lancet, 384(9940), p.329-336.

orabi, H., saba, I., Rousseau, A., & Bolduc, s. (2017). Novel three-dimensional autologous tissue-engineered vaginal tissues using the self-assembly technique. Translational Research, 180, p.22-36.

ojha, s. s., stevens, D. R., Hoffman, T. J., stano, K., Klossner, R., scott, M. C., ... & Gorga, R. E. (2008). Fabrication and characterization of electrospun chitosan nanofibers formed via templating with polyethylene oxide. Biomacromolecules, 9(9), p.2523-2529.

ohkawa, K., Cha, D., Kim, H., Nishida, A., & yamamoto, H. (2004). Electrospinning of chitosan. Macromolecular rapid communications, 25(18), p.1600-1605.

Qin, y., Liu, D., Wu, y., yuan, M., Li, L., & yang, J. (2015). Effect of PLA/PCL/cinnamaldehyde antimicrobial packaging on physicochemical and microbial quality of button mushroom (Agaricus bisporus). Postharvest biology and technology, 99, p. 73-79.

Rose, W. A., McGowin, C. L., spagnuolo, R. A., Eaves-Pyles, T. D., Popov, V. L., & Pyles, R. B. (2012). Commensal bacteria modulate innate immune responses of vaginal epithelial cell multilayer cultures. Plos one, 7(3) , e32728.

Rijal, N. P., Adhikari, U., & Bhattarai, N. (2017). Production of electrospun chitosan for biomedical applications. In Chitosan Based Biomaterials Volume 1 (pp. 211-237). Woodhead Publishing.

Rennert, K., steinborn, s., Gr6ger, M., Ungerb6ck, B., Jank, A. M., Ehgartner, J., ... & Mosig, A. s. (2015). A microfluidically perfused three dimensional human liver model. Biomaterials, 71, p.119-131.

sacchi, M. & Bansal R. (2020). Bioengineered 3D Models to Recapitulate Tissue Fibrosis. Trends in Biotechnology, 38(6), p.623-636.

shahrubudin, N., Lee, T. C., & Ramlan, R. J. P. M. (2019). An overview on 3D printing technology: Technological, materials, and applications. Procedia manufacturing, 35, p.1286-1296.

schiffman, J. D., & schauer, C. L. (2007). Cross-linking chitosan nanofibers. Biomacromolecules, 8(2), p.594-601.

shen, L., zhang, W., yuan, y., zhu, W., & shang, A. (2022). Vaginal microecological characteristics of women in different physiological and pathological period. Frontiers in Cellular and Infection Microbiology, 12, 959793.

sijbesma, R. A., van Waarde, A., & Elsinga, P. H. (2014). shivashankar Khanapur, soumen Paul, Siddesh Hartimath, Jurgen W. Development and Preclinical Evaluation of Radioligands for the PET Studies of Cerebral Adenosine A1 and A2A Receptors, 161.

Tomas, M. (2020). Bacterial vaginosis: Standard treatments and alternative strategies. International Journal of Pharmaceutics, 587(25), p.119-129.

Tucker, N. (2015). The development of electrospinning technologies for commercial application.

Tantengco, O. A. G., Richardson, L. S., Radnaa, E., kammala, A. k., kim, S., Medina, P. M. B., ... & Menon, R. (2022). Modeling ascending Ureaplasma parvum infection through the female reproductive tract using vagina-cervix-decidua-organ-on-a-chip and feto-maternal interface-organ-on-a-chip. FASEB journal: official publication of the Federation of American Societies for Experimental Biology, 36(10), e22551

Tuzlakoglu, k., Alves, C. M., Mano, J. F., & Reis, R. L. (2004). Production and characterization of chitosan fibers and 3-D fiber mesh scaffolds for tissue engineering applications. Macromolecular Bioscience, 4(8), p.811-819.

Tien, N. D., Geng, T., Heyward, C. A., Reseland, J. E., Lyngstadaas, S. P., Blaker, J. J., & Haugen, H. J. (2022). Solution blow spinning of highly deacetylated chitosan nanofiber scaffolds for dermal wound healing. Biomaterials Advances, 137, 212871.

valizadeh, A., & Mussa Farkhani, S. (2014). Electrospinning and electrospun nanofibres. IET nanobiotechnology, 8(2), p.83-92.

vaz, C. M., van Tuijl, S., Bouten, C. v. C., & Baaijens, F. P. T. (2005). Design of scaffolds for blood vessel tissue engineering using a multi-layering electrospinning technique. Acta biomaterialia, 1(5), p.575-582.

WANG W, ZHAO J W, YAO Z X, LIU J Z, SHI Z M, LI Y S, ZOU J, RUAN H J.(2020.) Oriented inner fabrication of bi-layer biomimetic tendon sheath for anti-adhesion and tendency healing.11,p.1-8.

Wolter, J. R., & kunkel, S. L. (1985). Artificial anterior chamber made of rigid PMMA contact lenses. Eye & Contact Lens, 11(2), p.107-110.

Wan, F., & Yang, M. (2016). Design of PLGA-based depot delivery systems for biopharmaceuticals prepared by spray drying. International journal of pharmaceutics, 498(1-2), p.82-95.

Wang, J., & Ye, L. (2015). Structure and properties of polyvinyl alcohol/polyurethane blends. Composites Part B: Engineering, 69, p.389-396.

Wu, Q., Liu, J., Wang, X., Feng, L., Wu, J., zhu, X., ... & Gong, X. (2020). organ-on-a-chip: Recent breakthroughs and future prospects. Biomedical engineering online, 19, p.1-19.

Xue, J., Wu, T., Dai, Y., & Xia, Y. (2019). Electrospinning and electrospun nanofibers: Methods, materials, and applications. Chemical reviews, 119(8), p.5298-5415.

Yi, X., Liu, F., Gao, K., Chen, F., Wang, Y., Li, H., ... & Gao, Y. (2022). Reconstructable uterus-Derived Materials for uterus Recovery toward Efficient Live Births. Advanced Materials, 34(8), 2106510.

Yi, H. G., Lee, H., & Cho, D. W. (2017). 3D printing of organs-on-chips. Bioengineering, 4(1), 10.

zhao, Y., Landau, s., okhovatian, s., Liu, C., Lu, R. X. z., Lai, B. F. L., ... & Radisic, M. (2024). Integrating organoids and organ-on-a-chip devices. Nature Reviews Bioengineering, 2(7), p.588-608.

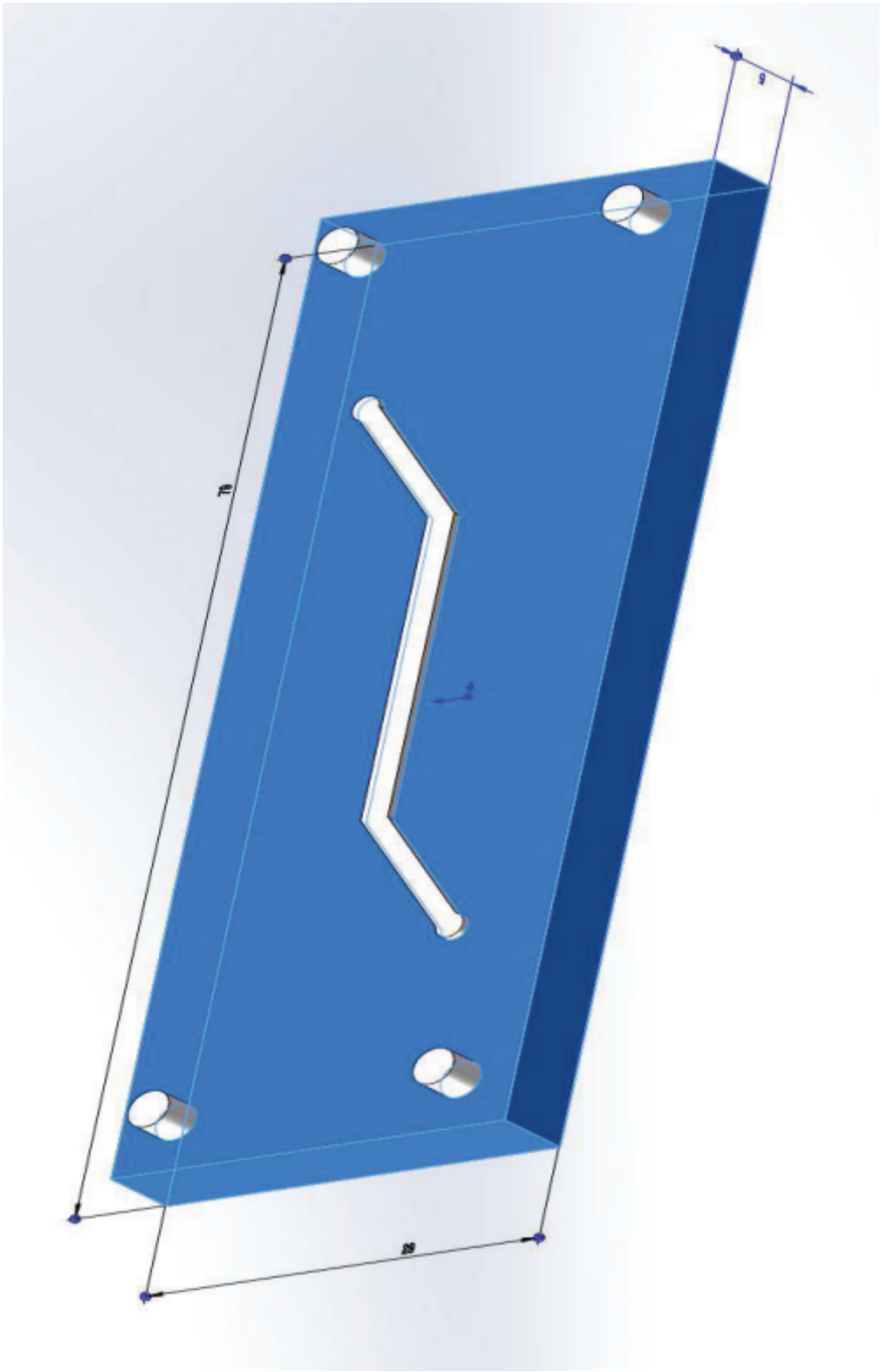
zhou, H., Lawrence, J. G., & Bhaduri, s. B. (2012). Fabrication aspects of PLA-CaP/PLGA-CaP composites for orthopedic applications: a review. Acta biomaterialia, 8(6), p.1999-2016.

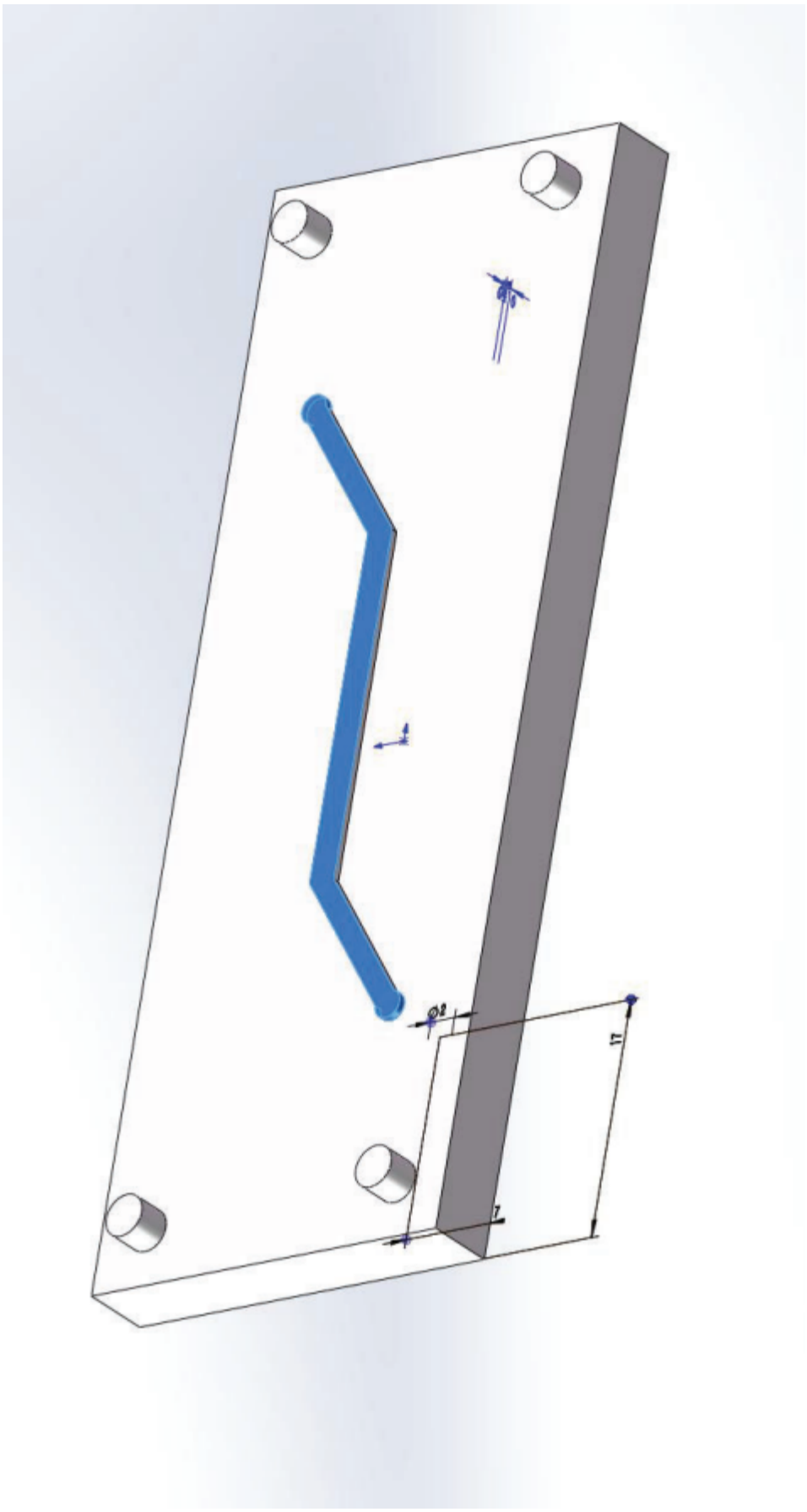
zhu, B., Bai, T., Wang, P., Wang, Y., Liu, C., & shen, C. (2020). selective dispersion of carbon nanotubes and nanoclay in biodegradable poly (ε-caprolactone)/poly (lactic acid) blends with improved toughness, strength and thermal stability. International journal of biological macromolecules, 153, p.1272-1280.

zou, Q., Grottkau, B. E., He, z., shu, L., Yang, L., Ma, M., & Ye, C. (2020). Biofabrication of valentine-shaped heart with a composite hydrogel and sacrificial material. Materials science and Engineering: C, 108, 110205.

9. Appendix A

9.1 Voc Bottom Layer Design Dimensions

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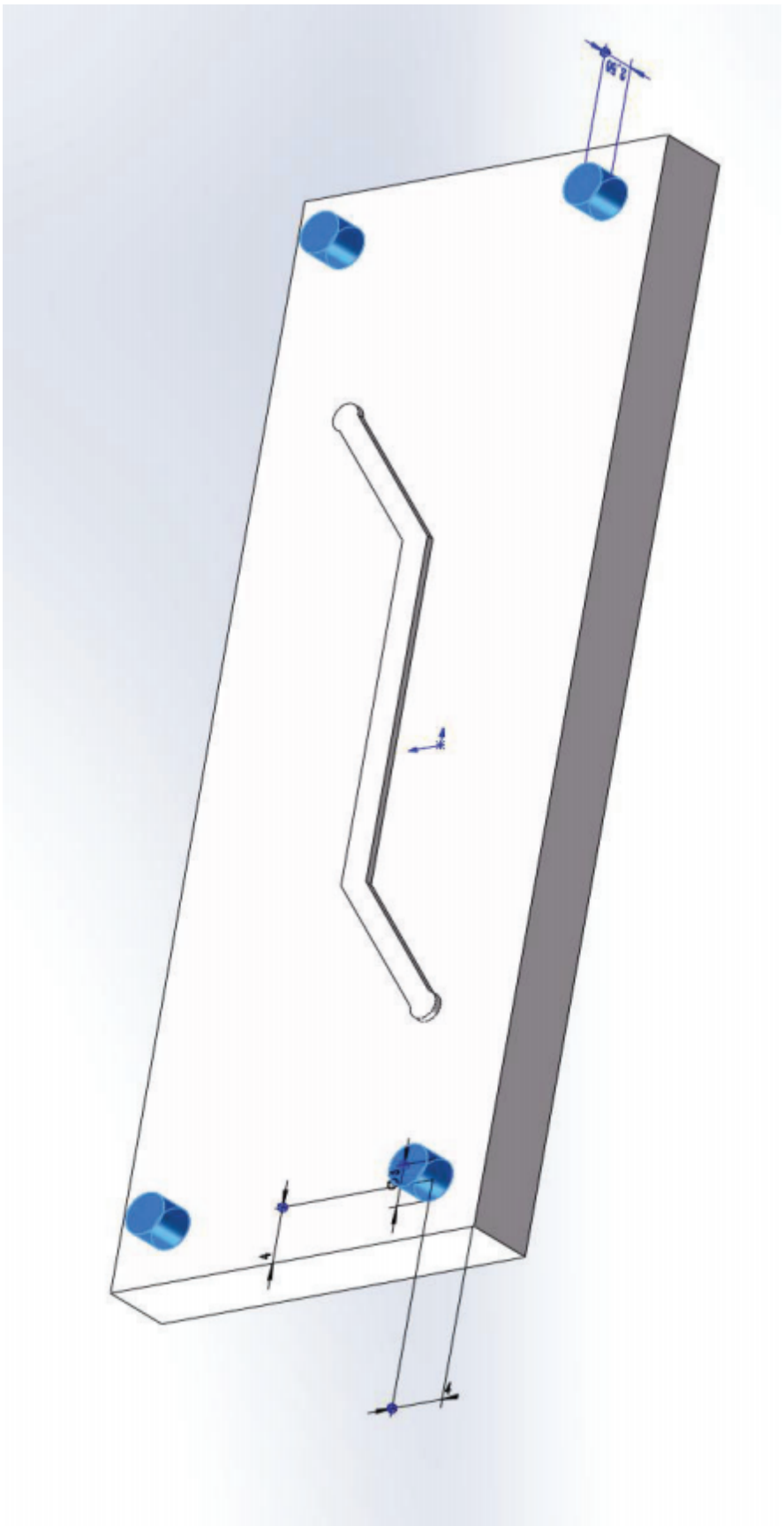

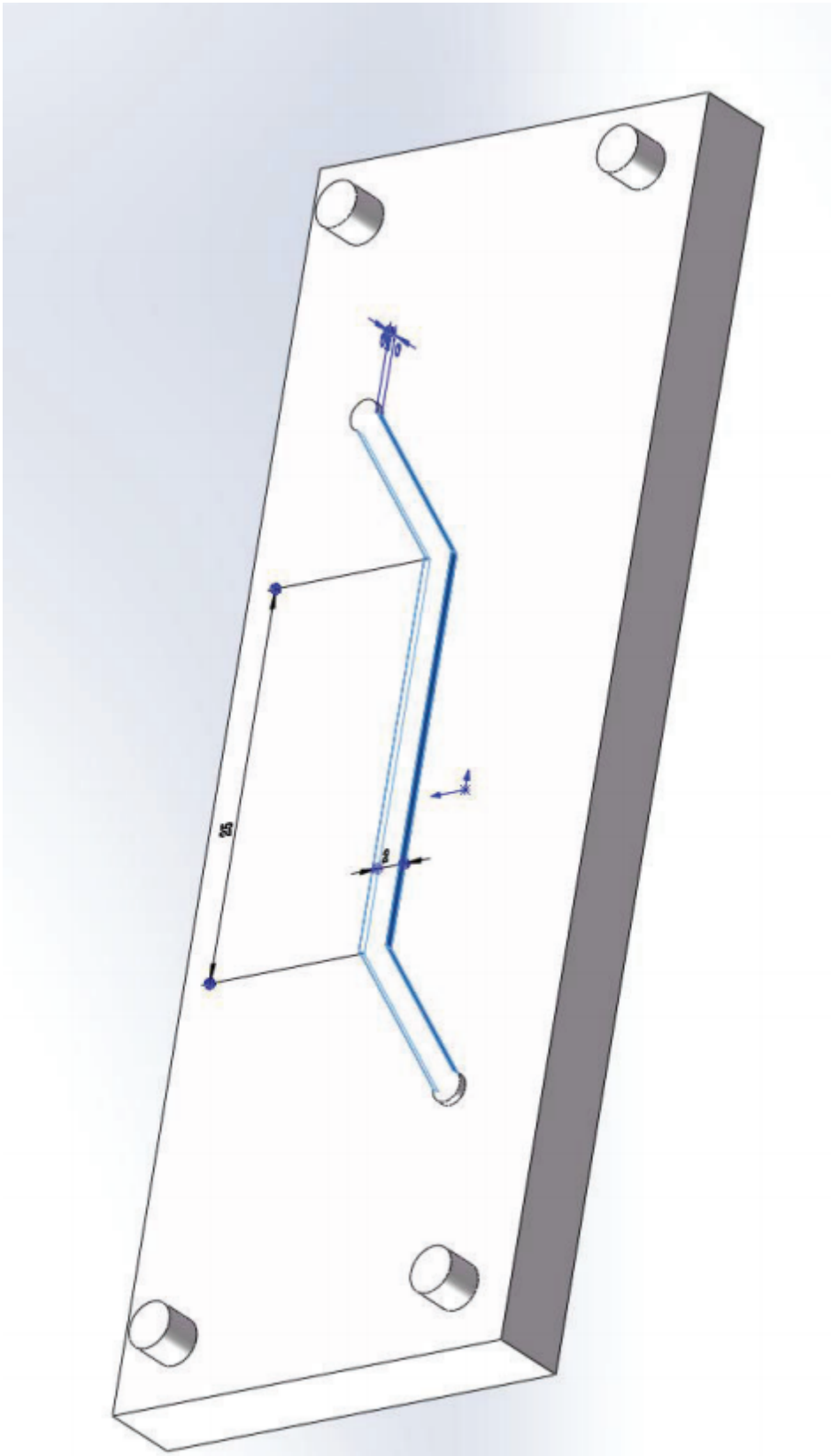
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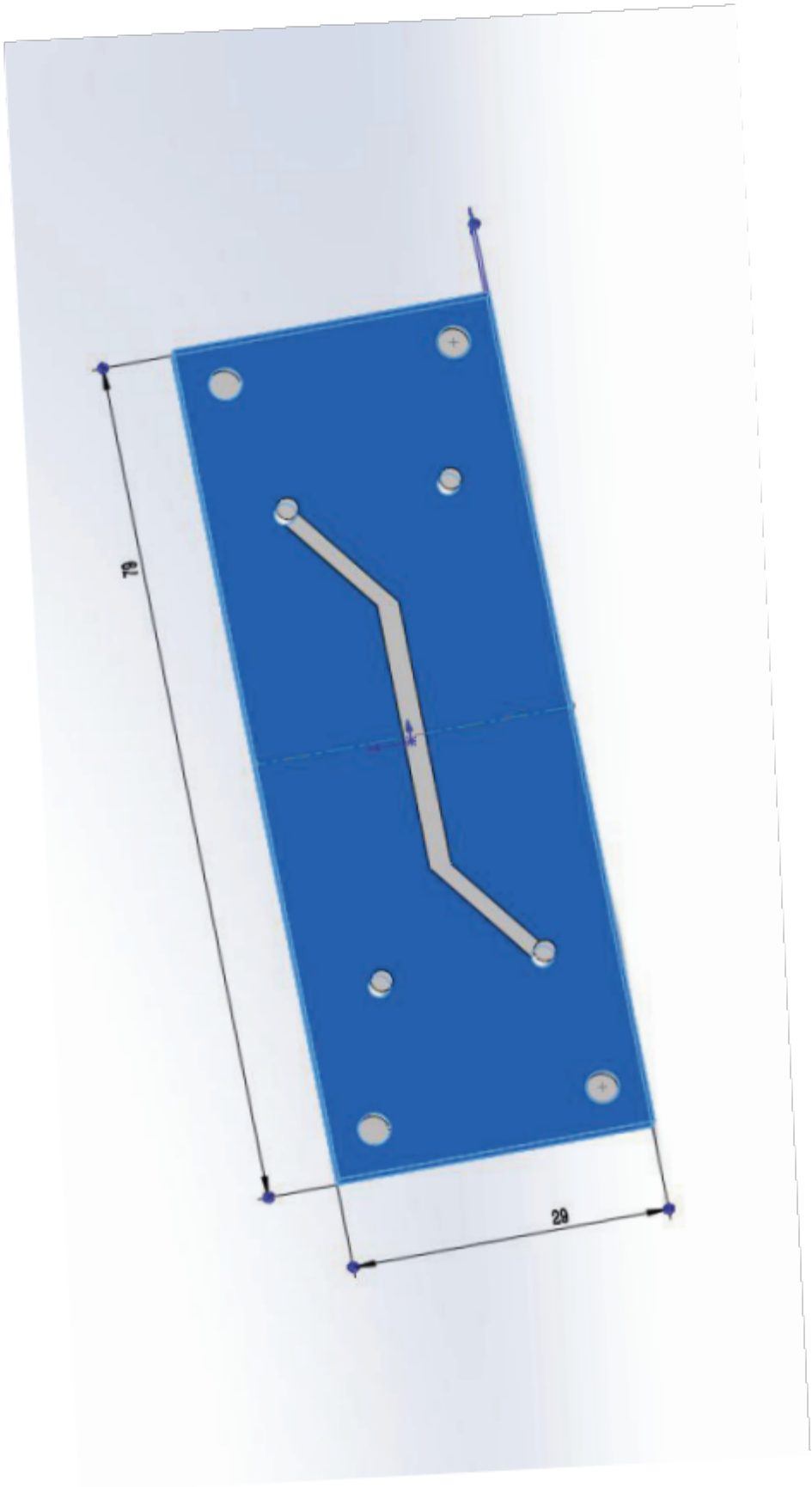
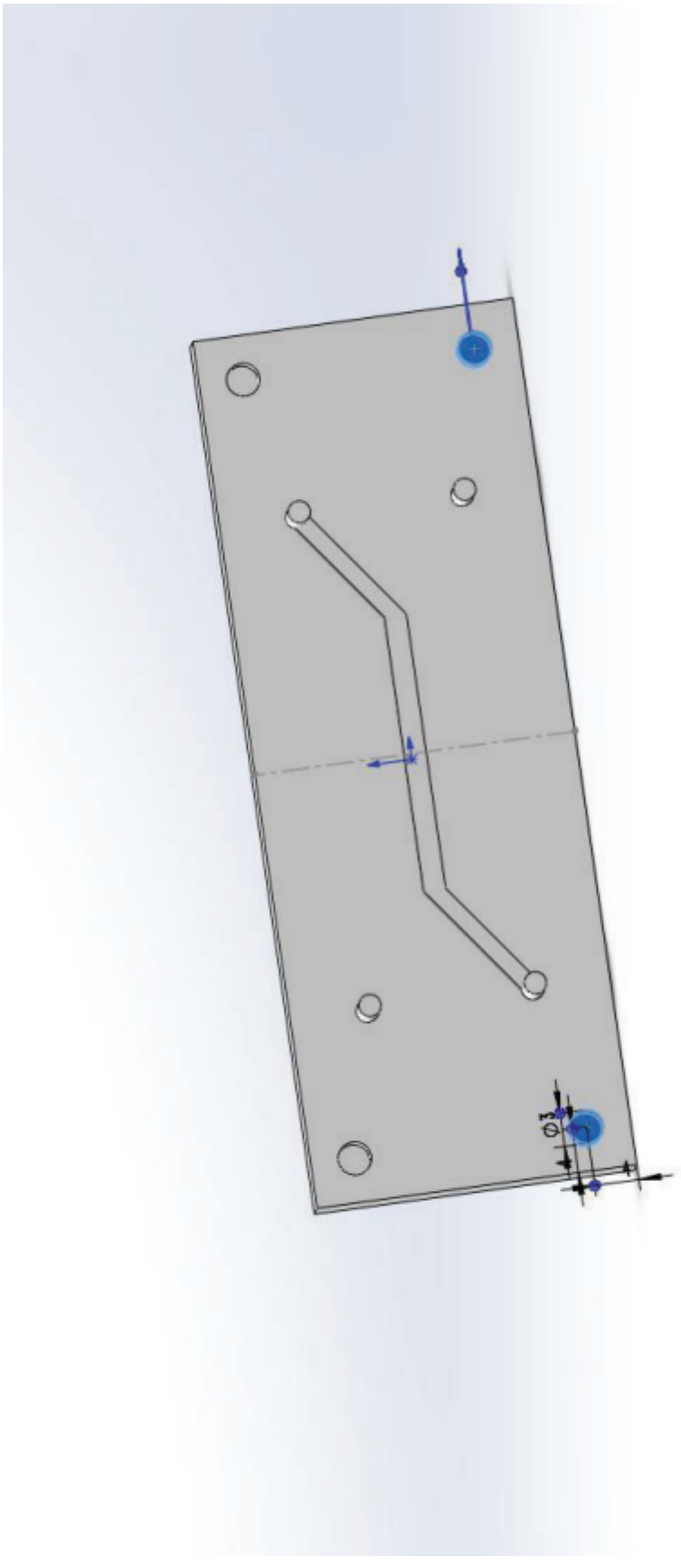
9.2 VOC ToP Layer Design Dimensions

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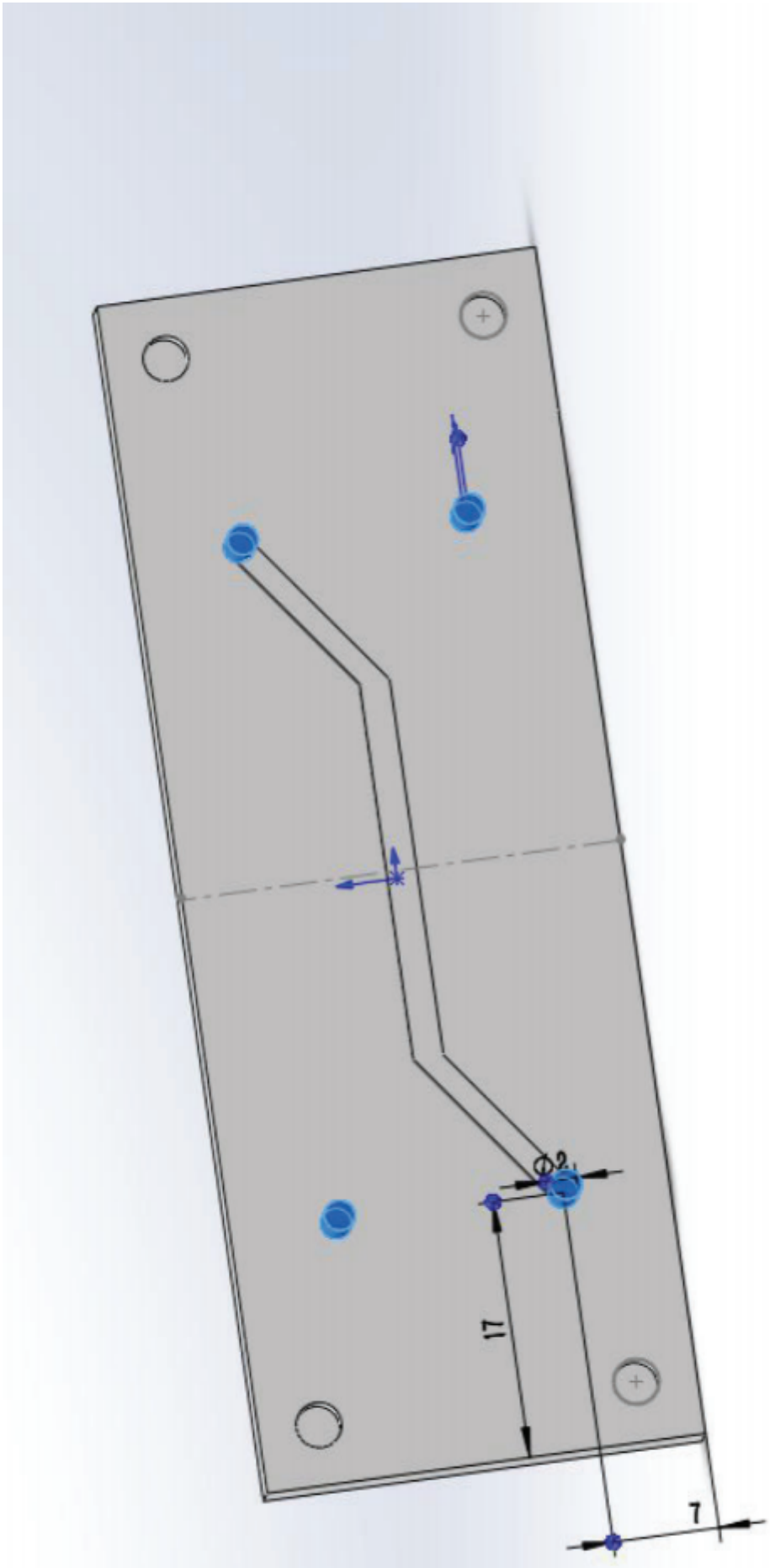
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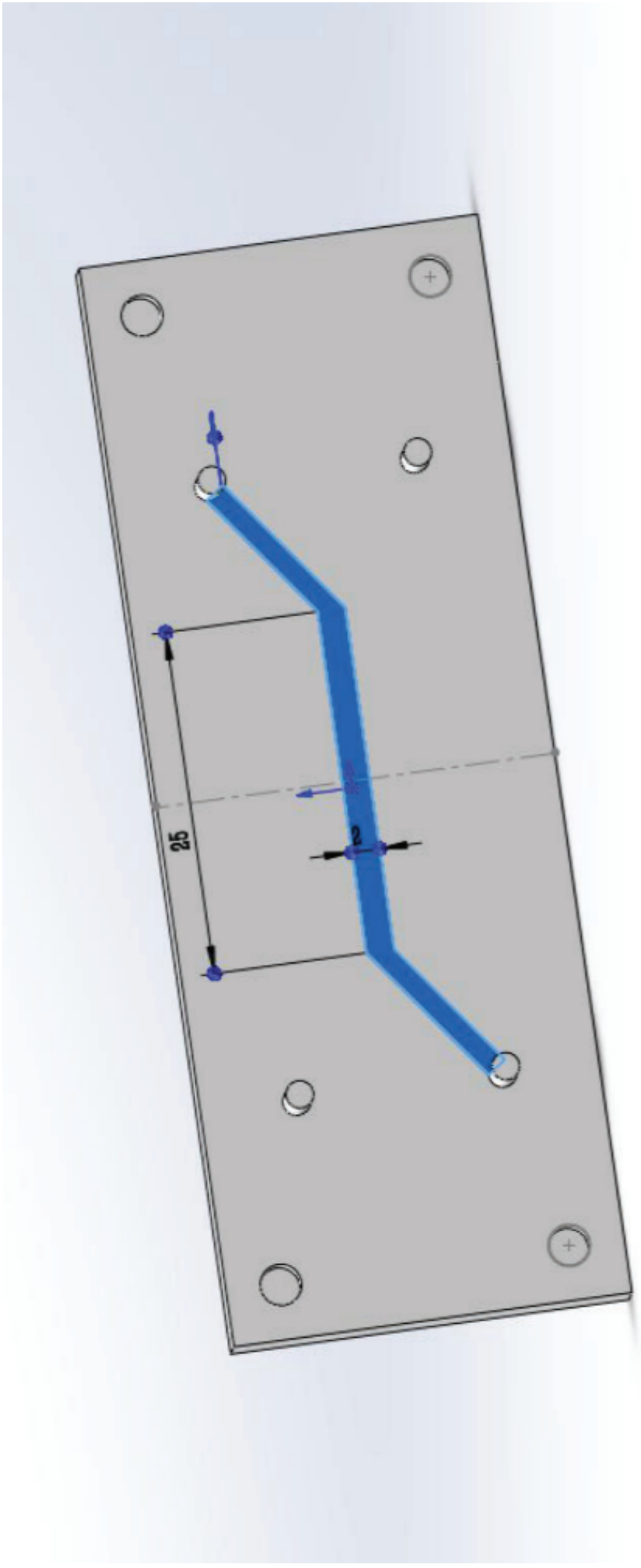
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