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Gelatin-Fibroin Sponges as Scaffolds in Cancer Tissue Engineering

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Abstract

Cancer tissue engineering using three-dimensional (3D) cell culture scaffolds has been reported to handle the limitations of two-dimensional (2D) cell culture in vitro and in vivo cancer models. In this paper, the scaffolds were produced via the freeze-drying technique and chemical cross-linking. 1-ethyl-3(3-dimethylamino propyl) carbodiimide hydrochloride (EDC) has been used as a crosslinker for conjugation between gelatin and silk fibroin (SF). The gelatin-fibroin (GF) scaffold evaluated the physicochemical characteristics such as microstructure, functional groups, swelling rate, degradability as well as some biological characteristics including in vitro cytotoxicity,for physicochemical characteristics such as microstructure, functional groups, swelling rate, degradability, and some biological characteristics including in vitro cytotoxicity and cell adhesion, cell proliferation and the spheroid forming capacity of MCF-7 cells on this scaffold. The results showed that the GF scaffold with the uniform pore size demonstrated suitable physicochemical properties that controlled swelling and degradation characteristics. The GF scaffold had no toxicity on human fibroblast cells (hF), and supported MCF-7 cells' adhesion, proliferation, and spheroid formation. In conclusion, this study successfully considered the potential of the GF scaffold as an in vitro 3D cell-culture scaffold in cancer tissue engineering in particular and tissue engineering in general.

Keywords: gelatin, silk fibroin, scaffold, cancer tissue engineering

1. Introduction

According to the GLOBOCAN 2020 estimates of cancer incidence and mortality provided by the International Agency for Research on Cancer, cancer is considered the leading cause of death and the main cause of reducing life expectancy worldwide. From 2015 to 2020, Breast cancer was commonly diagnosed cancer worldwide(Rashan *et al*., 2018; Youssry *et al*., 2019; Al-Momany *et al*., 2020; Sung *et al*., 2021; Ferlay *et al*., 2021). This shows the urgency in studying tumor formation, tumor invades and metastasizing, and the primary cause of cancer-related death. Human tumors consist of many different types of cells, such as cancer cells, fibroblasts, immune cells, etc., which function and are affected differently by many factors such as chemical, physical, and biological factors in the microenvironment. These components contribute to the growth of different regions within the same tumor (Thoma *et al*., 2014). However, the production of therapeutic drugs has encountered difficulties when entering the clinical trial stage because a suitable drug test model similar to tissue mass in vivo has not been found (Ibrahim *et al*., 2010; Aliwaini *et al*., 2020).

Typically, most of the experimental experiments were done in 2D cultures. Cells in the human body grow in an organized 3D matrix, surrounded by other cells. Individual cells are examined through their authors with neighboring cells and extracellular matrix (Smalley *et al*., 2006; Castells-Sala *et al*., 2013; Ha *et al*., 2013; Wu *et al*., 2014; Trivedi *et al*., 2021). Mono-layer culture in cancer research is a simple model that can be performed with many different concentrations and cell lines. If cultured for a long time, it will affect the cells inside.

Therefore, researchers only use mono-layer cell cultures to carry out simple studies in a short, not long, time. A significant drawback of this method is the misalignment of cell-cell, cell-ECM interactions, leading to the failure to form a tissue-like structure in the body. Since the tissue mass does not have the same system as the body's tissue mass, it will significantly affect the evaluation of the effectiveness of cancer treatments, therapies, and drugs. Given the limitations of mono-layer culture, animal models are quite common in the study of tumor growth and drug screening trials. Using animals in research, especially rats, often has the advantages of genetic similarity with humans, small size, ease of manipulation, and short reproduction time to observe the process performance. There are three types: mouse tumor-bearing mice, immunocompromised mice carrying human

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List of abbreviations : Silk fibroin SF; Gelatin-fibroin GF ;Human fibroblast HF

tumors, and humanized mice bearing human tumors. Besides the advantages, there are also many disadvantages when investing in research using this model is relatively high. The immune response and growth factors in mice differ from humans. Although the animal body carries a human tumor, that tumor is still modified to fit the animal body. However, the issue of bioethics is one of the leading issues when using this model.

One of the simplest 3D tissue mass models is the cluster cell consisting of the hanging drop and hydrogel methods to generate spherical tumors. Using gels to model cell clusters that can simulate the extracellular matrix, spherical tumor structures also helps simulate cell-cell, cell-ECM interactions, and extracellular signals of a mass of tissue in vivo. However, the use of this gel suffers from the disadvantages of biodegradability and porosity, which may affect cell distribution (Tung *et al*., 2011). Therefore, creating a suitable drug test model will speed up the drug testing process and reduce production costs.

Tissue engineering, which overcomes the limitations of traditional methods, offers new treatment opportunities for several diseases and is a new method for modeling human physiology. To restore a new tissue by tissue engineering, three components are required: cells, biological material (scaffolds), and bioactive molecules. Among them, biomaterials (scaffolds) play a significant role. The scaffold substrates can be highly efficient in loading and delivering cells to specific sites. Therefore, the scaffolds play an essential role in protecting and facilitating the interaction of the cell with the surrounding environment (Ma *et al*., 2005).

Recently, the combination of gelatin and fibroin has been examined for creating a new scaffold with suitable properties for cell culture and overcomes the disadvantages of both gelatin and fibroin. The gelatin-fibroin scaffolds have a porous structure, are highly biodegradable, non-toxic to cells, and support cell adhesion, and proliferation. When combined with cell culture, the tissue will be produced. The in vitro 3D cancer tissue mass is similar to the in vivo tissue mass (Jetbumpenkul *et al*., 2012; Lu *et al*., 2010; Petrenko *et al*., 2011; Asuncion *et al*., 2016; J *et al*., 2015; Pan *et al*., 2016; Dong *et al*., 2019; Nguyen-Thi *et al*., 2018; Nguyen *et al*., 2018).

This study aims to create gelatin-fibroin (GF) sponges based on the freeze-drying technique and chemical cross-linking EDC. The GF was generated with suitable properties as a scaffold for application in the cancer tissue engineering on microstructure, functional groups, swelling rate, and degradation properties; and investigated for its effects on hF in terms of cytotoxicity, cell adhesion, cell proliferation, and the spheroid forming capacity of MCF-7 cells on this scaffold.

2. Materials and Methods

2.1. Creating GF scaffolds

Fibroin solution from silk used for this study was provided by TEBM lab, University of Science, VNU-HCM. The solution of 3.5% fibroin and 0.24% EDC (Sigma-Aldrich, USA) with a ratio of 8:1 (v/v) was mixed, left for 15 minutes at room temperature, and then added 9% gelatin (Merck, USA) with a ratio of 9:8 (v/v) to the above mixture, left for 2 hours at room temperature. Gelatin-fibroin mixture was frozen at -86°C (Panasonic, Japan) overnight and freeze-dried (SP Scientific, England) at 100 mT pressure, -73°C temperature for 12 hours. Sponges were washed with distilled water for 7 hours, changed the water every 20 minutes. The soaked sponges were frozen at -86°C and freeze-dried a second time with a pressure of 100 mT and a temperature of -73°C for 8 hours. Finally, these sponges were sterilized by gamma irradiation dose 25kGy and evaluated some characteristics as scaffolds in tissue engineering.

2.2. Structure of scaffolds

Images were taken using an inverted microscope (Olympus, Japan) equipped with a DP2-BSW digital camera, and a scanning electron microscope (SEM, JSM-6510, JEOL, Japan).

2.3. Functional groups

The chemical compounds of the GF scaffold were identified by PerkinElmer Fourier transform infrared (FTIR) and diode array (DA) spectrometers (Nicolet 5700, USA).

2.4. Swelling rate

According to BS EN 13726-1:2002 standard, the change of a GF scaffold weight in solution A (8.298g of sodium chloride and 0.368g of calcium chloride dihydrate in deionized water and making up to 1 liter) after incubating at 37°C for 30 minutes, was used to observe the swelling rate (%):

Swelling rate (%) = (W1 -W0)/W0 x 100%,

W0 is the weight of the GF scaffold, and W1 is the weight of the GF scaffold after incubating in solution A.

2.5. Degradation rate

The weights of GF scaffold before (D) and after incubating in PBS (Gibco, USA) solution at 37°C; then dried for 15 minutes at 60°C and weighed at 1; 2; 3; 4; 5; 6; 7; 8; 9 days (L) were used to calculate the degradation ratio as per the equation:

The degradation ratio (%) = (D - L)/D x100%

2.6. In vitro cytotoxicity

Human fibroblast cells (hFs) used for cytotoxicity testing are available in the Laboratory of Tissue engineering and Biomedical Materials. The level of cytotoxicity of GF scaffolds was performed by measuring the relative viability of this according to the ISO 10993-5:2009 instruction. The test sample (GF scaffold) and the positive control (latex) were incubated in a complete medium (DMEM-F12 (Sigma–Aldrich, USA), which supplemented with 10% Fetal Bovine Serum (FBS, Sigma–Aldrich, USA) and 1X antibiotics (Sigma–Aldrich, USA) at 37°C for 24 hours. The negative control was the complete medium. The GF and latex were collected and placed on the subconfluent monolayer of the cells and incubated at 37 °C for 24 hours. The forms of cells were captured with an inverted microscope equipped with a DP2-BSW microscope digital camera.

2.7. Cell seeding

MCF-7 cells ($1.5x10^4$cells per milliliter) were seeded into a GF scaffold of 3x3x3mm by incubating in the

culture medium at 37 °C, 5% CO_2 for 24 hours. The non-adherent cells were collected and used to calculate the cell seeding efficiency as per the formula:

The cell seeding efficiency (%) = (Total cells were seeded into scaffolding - The non-adherent cells)/Total cells were seeded into scaffold x 100%

2.8. Cell proliferation

MCF-7 cells were cultured in the GF scaffold at 37 °C, 5% CO_2 for 0; 2; 4; 6; 8 days and evaluated proliferation by an in vitro DNS (3,5-dinitrosalicylic acid) assay. The culture medium was collected, and 100 µl of DNS (1%) (Sigma–Aldrich, USA) was added and mixed at 80 °C for 20 minutes. The color of this liquid was quantified by measuring the absorbance at 570 nm using a microplate reader (Biochrom, USA) (Pepper S. *et al*, 2006).

2.9. Histological evaluation

After seeding into the GF scaffold, MCF-7 cells were cultured in the scaffold with the culture medium at 37°C, 5% CO_2, and replaced the medium every 2 days. The MCF-7-GF scaffold complex was collected after 7 days cultured and fixed with 10% formalin at 4°C for 24 hours. This complex after dehydrating by ethanol and immersing in xylene was embedded in paraffin. These paraffin sections were cut at 4 µm, and stained with H&E (Hematoxylin and Eosin). The inverted microscope equipped with a DP2-BSW microscope digital camera was used to take a photo of this complex after staining.

2.10. Statistical analysis

Each treatment was repeated 3 times. GraphPad Prism version 8.0.2 (GraphPad Software, Inc., San Diego, CA) was used to perform all statistical analyses.

3. Results

3.1. Creating GF scaffolds

The GF scaffolds consisted of 3.5% fibroin, 9% gelatin, cross-linked by 0.24% EDC. Fig.1 showed many pores of the GF scaffolds that can allow cells to adhere inside.

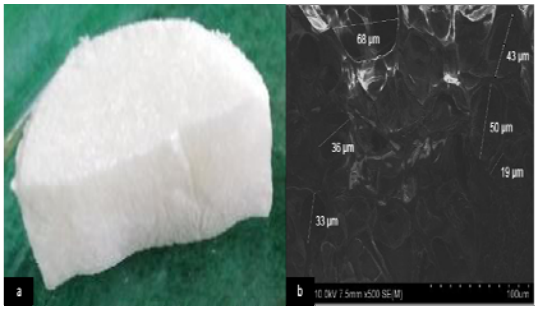

Figure 1. A GF scaffold (a), Structure of a GF scaffold under SEM (b)

3.2. Chemical structure of GF scaffolds

The FTIR spectra in Fig.2 showed an absorption peak at 1,637cm^{-1}, which belongs to the wavelength of the β-sheets form (1,620 – 1,640 cm^{-1}). Besides, the FTIR spectra also showed another absorption peak at 3,435cm^{-1}, which belongs to the wavelength of the form of the N-H bonds (3,200 – 3,400 cm^{-1}). This proved that the GF scaffolds also retain the distinctive properties of gelatin (Hermanto *et al*., 2013). In addition, another absorption peak belongs to the wavelength of the form of the CO-NH bonds (1,550 – 1,650 cm^{-1}), indicating that EDC cross-linked fibroin and gelatin. The FTIR spectra did not show any absorption peaks which belong to the wavelength of the form of the N=C=N bonds (2,120 – 2,145 cm^{-1}) significantly of EDC.

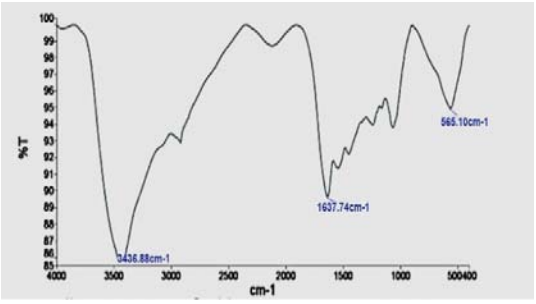

Figure 2.FTIR spectra of GF scaffolds

3.3. Swelling rate of GF scaffolds

The Fig.3 showed that the swelling rate of the GF scaffold is 714.7 ± 61.10% which is more than the swelling rate of scaffolds that use a glutaraldehyde cross-linking method (TK1, 400%) (Dong *et al*., 2019) and other scaffolds which do not use any cross-linking methods (TK2, 127%) (Lu *et al*., 2019). The swelling rates of the GF scaffolds, the glutaraldehyde cross-linking scaffolds and the non-cross-linking scaffolds have a statistical difference (P-value < 0.05).

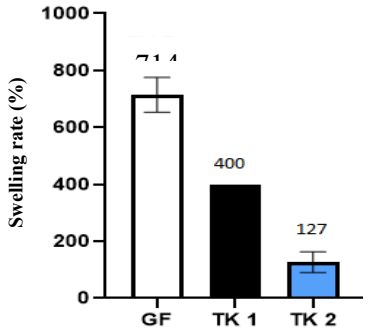

Figure 3. Swelling rate of GF scaffolds

3.4. Degradation rate of GF scaffolds

The degradability of GF scaffolds was investigated within nine days in PBS solution. The results showed that the GF scaffolds degraded by 11.4% by weight in the PBS solution in nine days, the scaffolds remained around 90% after seven days.

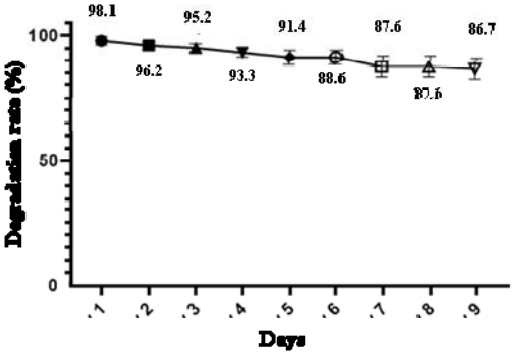

Figure 4. Degradation rate of GF scaffolds

3.5. In vitro cytotoxicity of GF scaffolds

The latex, employed as the positive control, caused severe cell death. Meanwhile, the cells had the standard appearance and could proliferate typically around and under the GF scaffolds. It could be concluded GF scaffolds did not cause toxicity to hFs that is level 0 according to ISO 10993-5. Therefore, the GF scaffolds could be used in cell culture.

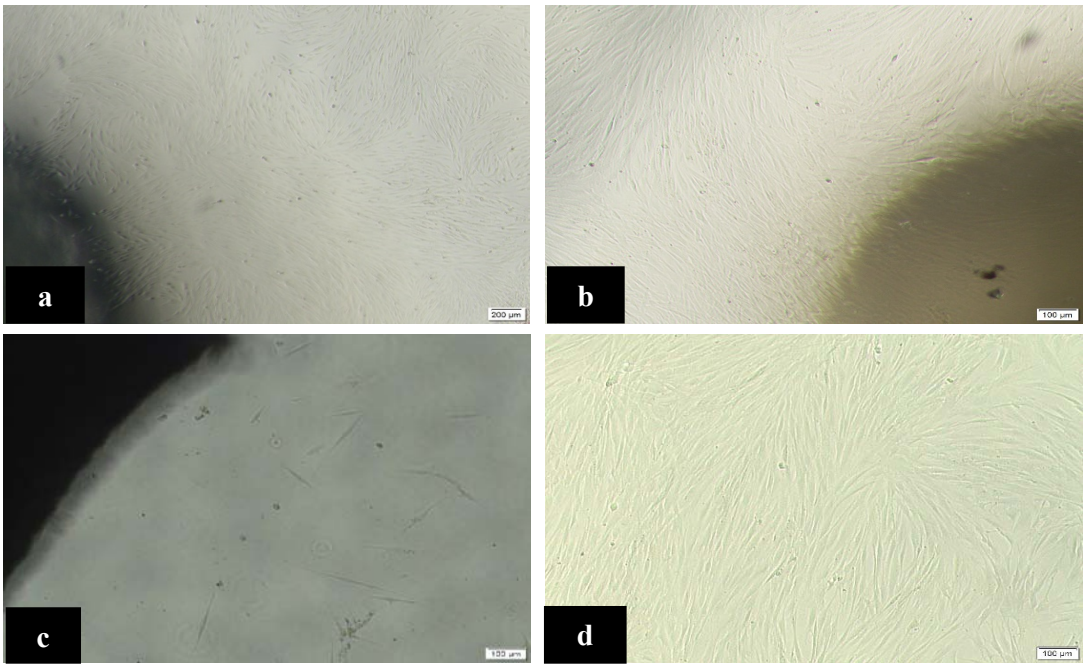

Figure 5. In vitro cytotoxicity of GF scaffolds (a, b), positive control (c), and negative control (d) (n = 3)

3.6. Cell proliferation in GF scaffolds

The MCF-7 cells could adhere and form spheres in the GF scaffolds. At 570 nm, the OD value is inversely proportional to the cell density (Fig.6). These results showed that the GF scaffolds could make the MCF-7 cells adhesion and proliferation. From D-0 to D-2, the OD value has no statistical difference. From D-0 to D-4, the OD value decrease and have a statistical difference (P-value < 0.001). However, from D-4 to D-8, the OD values were in the stable stage.

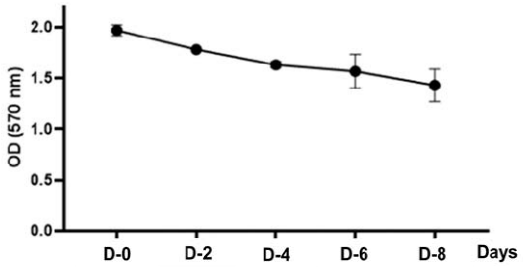

Figure 6. The OD value of the DNS experiment from D-0 to D-8

Fig.7 showed MCF-7 cells could adhere and form spheres inside GF scaffolds. These spheres increased from D-1 (d-31.82 µm) to D-7 (d-119.44 µm) while being cultured by the incubation method.

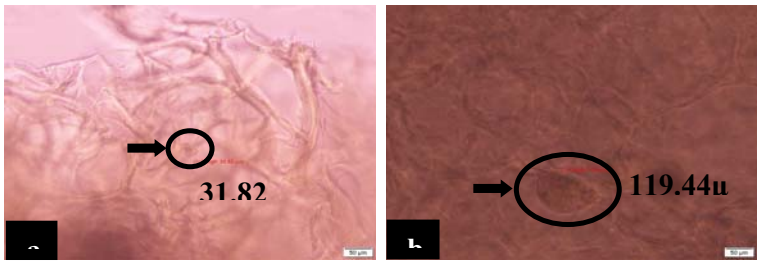

Figure 7. The MCF-7 cells in GF scaffolds on D-1 (a), and D-7 (b)

The MCF-7 cells were seeded into the GF scaffolds with a $1.5x10^4$ cells/mL density. The efficiency of this seeding process was 94.2%. The GF scaffolds could absorb the cell solution and keep them inside. After that, the cells could adhere stably to the GF scaffolds.

On D-7, the complex of MCF-7 cells and a GF scaffold included many cells in the extracellular matrix. Fig.8 showed that the nuclear area of MCF-7 cells got dark purple. The nucleus became clumped.

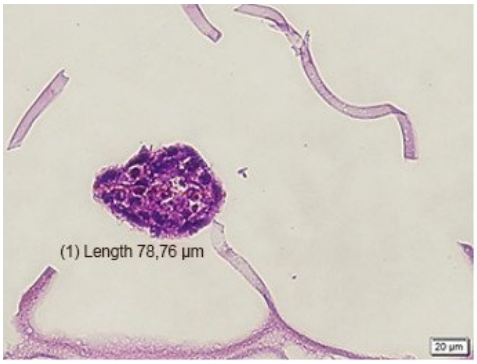

Figure 8. H&E staining of MCF-7 cells on D-7

4. Discussion

Cancer tissue engineering uses scaffolds for culturing cancer cells to study the proliferation, invasion, and metastasis of tumors. In addition, a three-dimensional scaffold is a temporary structure that supports cells to grow in each environment, which can eventually integrate to become tissue (Asghar *et al*., 2015). The supporting scaffold has a porous structure, the ability to provide adequate nutrients to meet the metabolic needs of the cell; it also supports the formation and distribution of vessels. Therefore, cancer tissue engineering has shown great potential when it is possible to simulate the mass of cancer tissue *in vivo* (Ehsan *et al*., 2014).

The combination of gelatin and fibroin by crosslinking not only inherits the superior properties of these two materials but also creates a new scaffold with suitable properties for cell culture and overcomes the disadvantages of both gelatin and fibroin (Jetbumpenkul *et al*., 2012, Wang *et al*., 2016). The scaffolds formed by pure fibroin solution could only make a layer-by-layer structure rather than a porous structure. The properties of durability and compression of scaffolds were lost. By supplementing gelatin in the mixture, the number of layers decreased, and more porous structures formed in the scaffolds because of the interaction of fibroin-gelatin, ultimately decreasing the tendency of the solid from the freezing liquid phase(Lu *et al*., 2010; Asuncion *et al*., 2016). The porous were formed while the ratios of fibroin-gelatin in weight were above 20%. The pore size depended on the concentration of fibroin and crosslinking method (Lu *et al*., 2010). The pore would be enlarged inside the crosslinked scaffolds because carboxyl side groups in gelatin are needed for fibroin-gelatin crosslinking by EDC (Asuncion *et al*., 2016). EDC molecules do not participate in cross-linking and rapidly degrade to non-toxic products, so EDC is less toxic to the body (Ha *et al*., 2013). The glass transition temperature (Tg) is the temperature at which the amorphous regions experience the transition from a rigid state to a more flexible state making the temperature at the border of the solid-state a rubbery state. This temperature in fibroin occurs between -20°C and -30°C, which affects the pore size of the GF scaffold. Faster freezing (-86°C) will reduce the formation of ice crystals inside the scaffold, so when freeze-drying, the GF scaffold has a small pore size with an average size of 100.6 ± 47.84 µm, which is very suitable for cell growth, allowing the diffusion of nutrients from the outside into the core of the scaffold and the removal of waste products. In addition, the FTIR spectra result with the absorption peaks at 1,637cm^{-1}, and 3,435cm^{-1} proved that the GF scaffold's structure is a combination of gelatin and fibroin (Hermanto *et al*., 2013, Kolev *et al*., 2017).

GF scaffolds were demonstrated to absorb and diffuse the culture medium nutrients into the scaffolds by the swelling rate. The holes structures of the GF scaffolds could increase the water absorption capacity of the scaffolds.

In the process of scaffolds creation, the α-helix forms and random coils forms reconstruct into β-sheets forms to achieve high-strength structures (Kolev *et al*., 2017), suitably for the degradable rate of GF scaffolds. The result is similar to another study (Jetbumpenkul *et al*., 2017), after incubating in PBS for 9 days, our GF scaffolds had a degradable rate of 88.6% the same as the results of Jetbumpenkul *et al*. (2012) after 7 days with the remaining weight being 90%. That is one of the essential characteristics of scaffolds which shows that the strength of the scaffold is sufficient to conduct *in vitro* studies.

We confirmed our GF scaffolds as a non-cytotoxicity scaffold regarding the *in vitro* on hF, and its ability to stimulate MCF-7 adhesion with the efficiency of this seeding process more than 90% while another research showed that the efficiency was 80% (Jetbumpenkul *et al*., 2012). The proliferation processes of the MCF-7 cells in the GF scaffolds were investigated by DNS. A yellow compound could be produced while the remaining glucose in the medium reacts with DNS. Because of the proliferation of the MCF-7 cells, the amount of glucose in the medium gradually decreases. The results of the DNS experiment and the microscope image showed that size of MCF-7 spheres increases while culturing by incubation for eight days. HE staining at 7 days showed that there were multiple cells bound together in a matrix scaffold.

However, the proliferation of the MCF-7 cells in the DNS experiment is not clear from day 4 to day 8 of cultured. Besides, this study required the co-culture of many cell types on the GF scaffold and increased incubation time to evaluate forming of a mass and determine the stage of the descriptor block to conduct drug testing. The results show that the GF scaffold is functional in 3D research *in vitro*.

5. Conclusion

The GF scaffolds were entirely created by freeze-drying and cross-linking with EDC. The pores of these scaffolds were 52.76 - 148.44µm. The average swelling rate was 653.6 - 775.8%. These scaffolds degraded by 11.4% by weight in PBS solution in nine days. GF scaffolds did not cause toxicity to fibroblasts. They could make the MCF-7 cell adhesion and proliferation. Therefore, the GF scaffolds could be used in cancer cell culture.

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