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Role of Co-Culture with Fibroblasts and Dynamic Culture Systems in 3-Dimensional MCF-7 Tumor Model Maturation

Nho Thuan Nguyen[1,2,3] , Vu Nguyen Doan[1,2,3] and Ha Le Bao Tran[1,2,3,*]

[1]*Laboratory of Tissue Engineering and Biomedical Materials, University of Science - VNUHCM, Ho Chi Minh City, Vietnam*
[2]*Department of Physiology and Animal Biotechnology, Faculty of Biology and Biotechnology, University of Science - VNUHCM, Ho Chi Minh City, Vietnam*
[3]*Vietnam National University, Ho Chi Minh City, Vietnam*

([*]Corresponding author's e-mail: tlbha@hcmus.edu.vn)

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Abstract

In vitro tumor models that are 3-dimensional (3D) have emerged as a significant area in the field of cancer research over the past several years. In order for breast cancer cell lines to grow and develop, it is important to select a scaffold, determine a strategy for seeding cells into the scaffold, then cultivate the cells under a variety of conditions. Therefore, we cultivated MCF-7 cells and fibroblasts on a gelatin-alginate scaffold alone or in co-culture under static or dynamic culture conditions in order to produce a 3D tumor model. MCF-7 and fibroblast were seeded into Gellatin-Alginate by Centrifugation and Incubation. After that, the cell proliferation was examined by MTT assay and the cell number determination in the scaffold. The morphology of MCF-7 was observed by H&E staining and SEM.

The results showed that the co-culture of MCF-7 cells and fibroblasts in the scaffold exhibited an increase in cell mass size. Their mass morphologies feature a significant number of MCF-7 cells with a round structure that persists for an extended period of time. Perfusion bioreactors also demonstrate an increase in the size of cell mass (3 times higher than static culture). As a result, the long-term stability of the structure offers the possibility of cancer biology research and drug testing, especially the sustained release or actions experiements.

Keywords: Perfusion bioreactor, Gelatin-alginate scaffold, Human fibroblast, Breast cancer cell line, Static culture, Dynamic culture

Introduction

Breast cancer is one of the leading causes of cancer-related death among females worldwide. So the study of breast cancer biology and the development of effective treatments are the current serious challenges. Although the conventional 2-dimensional (2D) cell culture technique was widely used to research cancer cells *in vitro* and for drug screening, it failed to test the new drugs due to mimicking the structure of the tissue *in vivo* [1]. In the principle of tissue engineering, the tumor *ex vivo* the combination of many elements: Cells, extracellular matrix (ECM), signaling factors, and their interactions. According to the culture models, cancer cell monolayer culture describes only one cell line without cell adhesion, interactions between cells and ECM, and interstitial fluid interactions, which were critical in cancer cells proliferation and differentiation [2]. In addition, although organ culture retains the features, it has difficulties in sustaining specimens and tissue viability over time. There are many drawbacks to using animals as models, such as high costs, extensive personal and work effort, and a short follow-up period [3].

Three-dimensional (3D) cell culture overcomes the limitations of 2-dimensional (2D) cell culture *in vitro* and in animal models due to 2 characteristics. Firstly, in tumor biology, the tumor-stromal interaction can provide a more realistic environment that elicits an *in vivo*-like response to cancer therapy. Secondly, 3-dimensional cultures more nearly mirror the form and environment of the cell, which determine the cell's character and gene expression [4]. However, 3-dimensional cell culture in the static culture is also hampered by difficulties in sustaining cultured cells, such as nutrient deficiency, external oxygen transfer, and waste disposal from cells within a framework. As a result, the culture is only maintained for a brief period of time; the cells inside the mass rapidly degenerate due to their incapacity to absorb vital chemicals, impairing the research that requires a lengthy formation and division process [5].

To limit the disadvantages, the system's perfusion-based bioreactors generate fluid flow and facilitate the movement of huge volumes of nutrients and oxygen throughout the supporting scaffolds. As the cell culture medium is pumped through the tube, it may flow in a controlled feedback loop that includes a reservoir and waste container. Oxygen is delivered to the system via silicon tubes or an oxygen generator, depending on whether air is brought in from the outside or generated on-site [6].

In addition to issues of culture system, it is also important to consider the material characteristics for the proliferation of cells. The available natural resources have already been used up. Gelatin is a naturally occurring polymer formed when collagen is partially hydrolyzed. Collagen is the most abundant protein in animals, constituting the majority of connective tissue. Due to gelatin's sensitivity to temperature fluctuations, it may be suited for layering via a sol-gel transition. Gelatin is practically more convenient than collagen since preparing a concentrated collagen solution from native collagen is highly laborious, and gelatin is also far less expensive. However, due to the limitation of gelatin's mechanical qualities, covalent crosslinking with alginate to increase the model's stiffness is the effective solution. Recently, gelatin and alginate were combined for bio-plotting, owing to their chemical resemblance to ECM [7]. Alginate is a harmless polymer found naturally in brown algae. Alginate is a hydrophilic substance that is biocompatible and relatively inexpensive. Native alginate hydrogels do not support cell adhesion but gelatin was primarily used to increase cell attachment. Furthermore, fibroblasts were added to the scaffold to increase its stiffness and breast cancer cell line proliferation. Adding many types of cell lines, on the other hand, requires an appropriate seeding solution to maximize the number of cells in the scaffold [8].

As a result, this study focuses on seeding approaches that enable the Gelatin-Alginate scaffold encasing human fibroblasts (hF) to hold a large number of cells. Following that, the mixture of MCF-7 and Gelatin-alginate scaffold was cultivated under static or dynamic conditions to maximize proliferation and structure efficiency.

Materials and methods

MCF-7 (Michigan Cancer Foundation-7) cell line and human fibroblasts were provided by Laboratory of Tissue Engineering and Biomedical Materials, University of Science, Vietnam National University, Ho Chi Minh City, Vietnam.

Preparation of gelatin-alginate scaffolds

Gelatin-Alginate scaffolds were prepared according to our previous publications [9, 10]. Briefly, gelatin (Sigma-Aldrich, USA) and alginate (Sigma-Aldrich, USA) were dissolved in distilled water at 50 °C to produce solutions with a concentration of 1 % gelatin and 1 % alginate, respectively. These mixtures were then combined with a volume ratio of 8 gelatin: 2 alginate and stored at −80 °C for 24 h. Following that, these frozen blocks were cross-linked in 0.3 % EDC (1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride) (Sigma-Aldrich, USA) for 24 h in a dark condition at 4 °C and then freeze-dried. The GA sponges were next sterilized using 25 kGy irradiation and sliced into pieces that were approximately 5×5×3 mm^3.

Cell maintenance

All cells utilized in this research were maintained and expanded in plastic flasks designed for cell culture. MCF-7 breast epithelial cells and human fibroblasts were cultured in DMEM/F12 (Sigma-Aldrich, USA) with 10 % FBS (Fetal Bovine Serum) (Sigma-Aldrich, USA). All cells were cultured at 37 °C and 5 % CO_2 in an incubator (Panasonic, Japan). Following that, cells that achieve 80 - 90 % confluence were used in the study.

Cell seeding

In this experiment, MCF-7 was used alone (1-cell samples) as well as in combination with fibroblasts (2-cell samples or co-cultured samples) to investigate the potential supporting role of fibroblasts. For MCF-7 samples, gelatin-alginate scaffolds were inserted into tubes before the media with 2×10^4 MCF-7 cells were added to each tube. The tube was incubated for 20 min in the cell incubator or centrifuged at 2,500 rpm for 2 min (Centrifuge machine Hettich, Germany). When the cell-scaffold complex was transferred to a new well, the medium in the old well was suspended, and assessed cell density in the medium. The more efficient solution will be employed in the following experiments.

For co-cultured samples, after culturing hF at a density of 0.5×10^4 cells/scaffold for 3 days, the MCF-7 line was continued at a density of 1.5×10^4 cells/scaffold.

Static culture method

The experiment was implemented with 2 samples. A single-cell sample of MCF-7 was grown in a Gelatin-Alginate scaffold. For 2-cell samples, hF was seeded into a gelatin-alginate scaffold before 3 days. After that, MCF-7 was introduced to the scaffold. An incubator set is 37 °C and 5 % CO_2 or both samples. In 2 samples, MTT assay (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (Sigma-Aldrich, USA) is used to measure the proliferation rate.

Dynamic culture method

After 4 days of static culture to stabilize the 2-cell complexes on a scaffold, it was transferred the culture plates to 2 dynamic culture systems. The first system consists of a culture dish fixed on an agitator with a speed of 100 rpm in 8 h/day. The medium was changed every 2 days. The second system is a perfusion bioreactor (Biotek, USA) with a flow rate of 6.04 cm/min. cultural time gets 24 h/day. The medium was changed every 10 days. Both treatments were evaluated in 28 days of culture. The cell proliferation rate of 2 experiments is examed by MTT assay.

Cell mass growth assay

After removing the culture media from the sample, MTT solution (5mg/mL) was added at the 9:1 ratio, cells were incubated at 37 °C and 5 % CO_2 for 4 h. The solution was discarded, DMSO/Ethanol and MTT/media solution (200 µl) were added to dissolve formazan crystals. The optical density (OD) was determined at 570 nm.

Cell morphology analysis

For this study, cell morphology in GA scaffolds was examined using Scanning Electron Microscope (SEM, S-3000N) and Hematoxylin and Eosin strainning (H&E). A cold D-PBS solution of 3 % Glutaraldehyde was used to fix the samples for 24 h. After that, the cell was dehydrated using a series of ethanol treatments. The specimens were preserved in 10 % formalin solution and embedded in paraffin for the H&E staining procedure. For morphological investigation, H&E staining was employed.

Statistics analysis

GraphPad Prism 7.0 was used to conduct the statistical analysis (GraphPad Software Inc., San Diego, CA, USA). P 0.05 was considered statistically significant. ImageJ version 1.52e was used to create the images (National Institutes of Health, Bethesda, Maryland, U.S.).

Results and discussion

There are numerous 3D breast cancer models available at the moment that incorporate the MCF-7 cell line in combination with scaffolds, gels, or basement membranes. However, it comes with several significant drawbacks, such as limited proliferation capacity, with MTT values consistently below 0.9 and the size blocks are not uniform in size. For exmaple, Lhuyen Fet al maintain the MCF-7 cell mass for 8 days and the highest MTT value is 0.9 [11]. The primary reason is that the framework does not adequately promote MCF-7 cell adhesion and growth. As a result, the experiment concentrated on assessing extracellular matrix components such as fibroblasts and gelatin.

Pan *et al.* evaluated cell adhesion with a concentration of 4×10^4 cells/ GA scaffold with dimensions of $10\times10\times2$ mm^3 in 500 µl of DMEM media, which showed promising results. As a result, a concentration of 2×10^4 cells/supporting framework $5\times5\times3$ mm^3 in 200 µl DMEM was used to evaluate cell adherence to the scaffold in this experiment. In addition, hF is the most prevalent cell type in the tumor microenvironment. These cells perform a variety of actions: They govern epithelial differentiation; regulate inflammation; produce parasecretory growth factors; proteolytic enzymes; include components of the extracellular matrix; promote tumor growth and metastasis. Excessive fibroblast accumulation and accelerated collagenization of the extracellular matrix are hallmarks of invasive breast cancer tissue, as documented by Kunz-Schughart LA. Thus, subsequent studies have focused on the co-culture of breast cancer epithelial cell lines with hF [7, 12].

Due to the mixing of 2 distinct cell lines, the seeding method used in the scaffold is critical for maintaining a sufficient number of cells in the scaffold. Static seeding or incubation is a common technique that involves placing cells and scaffolds in close proximity to promote cellular adhesion and migration inside the scaffolds. The centrifugal force approach is also used, and it makes it easier for cells to be transferred onto porous scaffolds in a homogeneous way. It was effective at low cell concentrations, used less disposable resources, introduced cells over the whole thickness of a particular scaffold, and shortened the time required to transition from cell seeding to cell proliferation [13].

After cultivating MCF-7 and hF cells in flasks to the desired density (cell confluence is 80 - 90 %), the seeding methods was studied for both experiments: (1) Incubation method 37 °C, 5 % CO_2 for 20 min; (2) Centrifugal method 1,500 rpm for 2 min.

Seeding performance

One-cell samples had a seeding rate of 94 % when implanted in the scaffold. When 2-cell samples were placed on the scaffold, the efficiency dropped to roughly 85 %. This could be due to the fact that adherent fibroblasts, seeded before 3 days, restrict the pores. It makes MCF-7 more difficult in entering the mass.

In comparison, centrifugation required minimal time in both trials, but its efficiency was rather low (about 40 %), cell mortality was high, and the distribution of cells on the support frame was not uniform (**Figure 1**). This demonstrates that the centrifugation speed and time are insufficient.

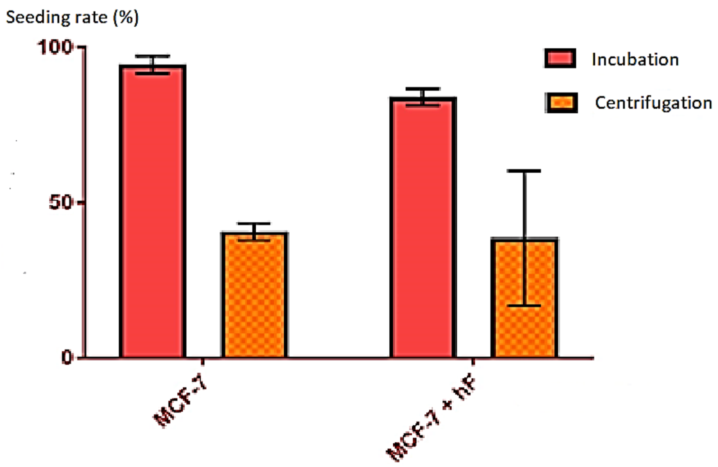

Figure 1 Cell seeding productivity for 1 or 2-cell sample.

During incubation, the solution containing the cells is absorbed and the cells are kept in a scaffold, after a suitable time the cells will stabilize in it. Most of the current tissue engineering studies use the incubation method to immobilize the cells on the scaffold and achieve many results, but the cell/frame efficiency is still not optimal. Godbey (2004) suggested that cells are not only affected by high centrifugation speeds, but also by speeds less than 2,000 rpm. The reason for this is that centrifugal force prevents the rotor from rotating in a balanced manner, which results in imbalanced rotation, vibration, and cell death. Additionally, the Godbey experiment showed that intermittent centrifugation for 5 times 1 min performed better than continuous centrifugation at 2,500 rpm for 1 and 5 min. As a result, we will increase the spin speed to 2,500 rpm to reduce the number of dead cells and perform interval rotation 5 times per minute to maximize efficiency [13].

Adhesion ability

On an inverted microscope, MTT-stained MCF-7 cells are likely recognized. The cells in the incubation method are significantly more than in the centrifugal solution (**Figure 2**). The SEM results also show identical results (**Figure 3**). When grown on Gelatin-Alginate scaffolds, MCF-7 had an epithelial-like shape with a cobblestone-like appearance. After the addition of MCF-7 and fibroblasts, SEM revealed the presence of a rough and uneven matrix on the scaffold surface, whereas uncellularized part revealed a smooth and regular one. It is demonstrated that the macroporous GA scaffold boosted cancer cell adherence, growth, proliferation, and particularly penetration into the macroporous scaffold for at least 300 nm. As a result of these observations, it was concluded that scaffolds made of GA with an appropriate pore shape make effective culture media for cancer cells.

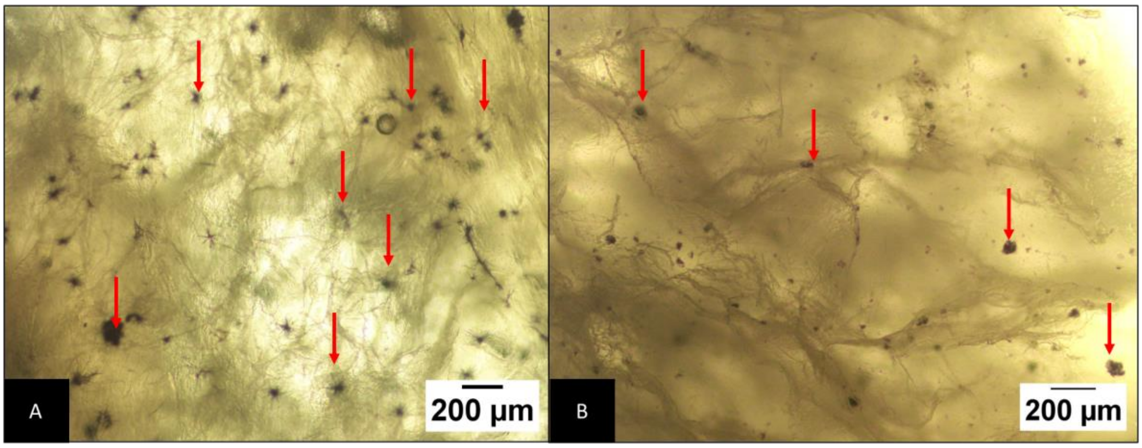

Figure 2 The distribution of MTT-stainned MCF-7 in the scaffold after seeding (A. Incubation, B. Centrifugation). **The red arrows** show the cells in the GA scaffold.

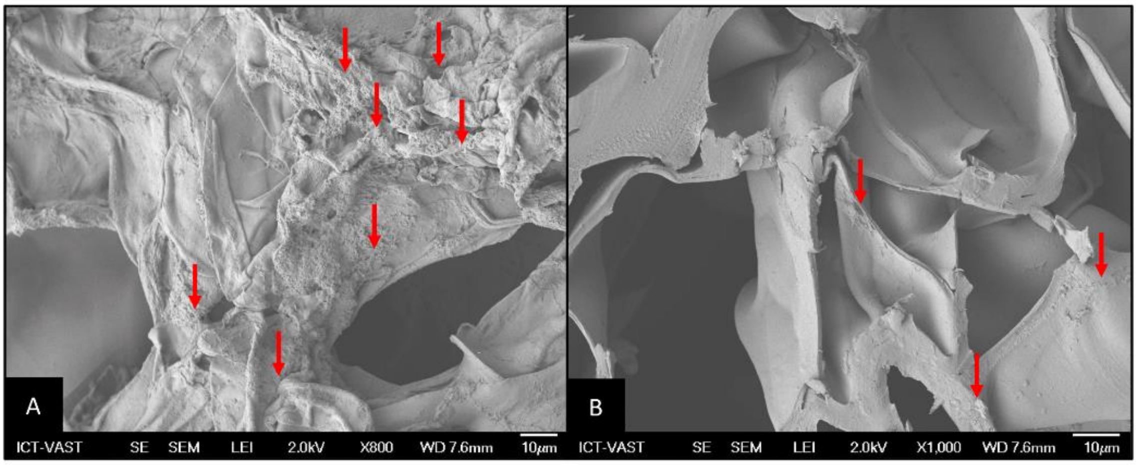

Figure 3 The MCF-7 distribution on Gelatin-Alginate scaffold (A. Incubation, B. Centrifugation). **The red arrows** show the cells in the GA scaffold.

Evaluation of the proliferative capacity of the cell mass in static culture

The proliferation of the MCF-7 cell line on the scaffold was studied using the MTT assay at periods from 0 to 10 days after seeding.

In **Figure 4**, cells proliferated till day 6 in a 1-cell sample before declining until day 12 while cells grew until day 8 in a 2-cell sample, stabilized at day 10, and subsequently declined at day 12 ($p < 0.05$). It took cells longer to proliferate than the 1-cell sample, but this proliferation remained steady until 8 days after MCF-7 was introduced, and then began to decline rapidly. This shows a more stable of 2-cell sample and the fibroblast's supporting role in MCF-7 growth. The high peak of a 1- and 2-cell samples are nearly identical.

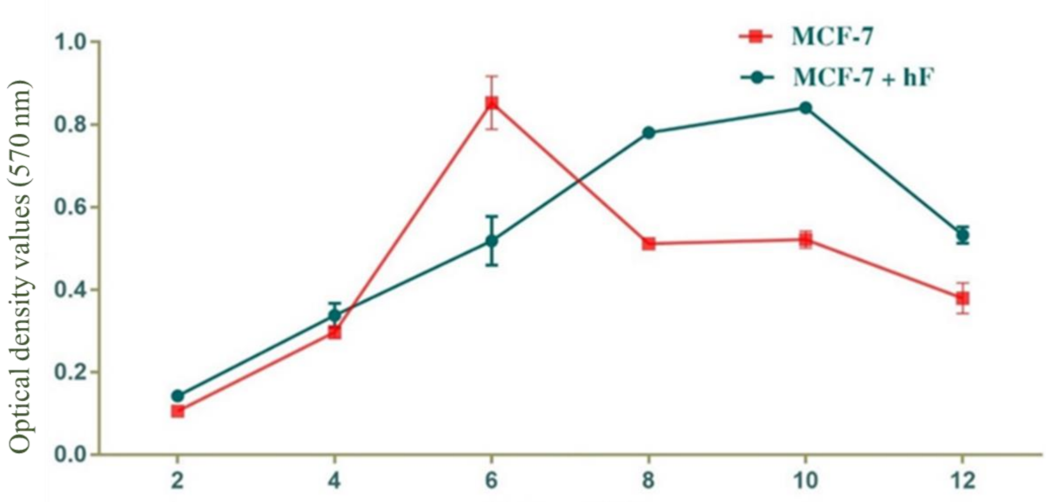

Figure 4 The proliferative ability of 1- or 2-cell samples in 12 days in static culture method.

Dhiman *et al.* (2005) measured the sugar concentration in the medium to test MCF-7 cell growth on a chitosan scaffold. After 5 days of culture, MCF-7 proliferated on the support frame. In this experiment, MCF-7 cells proliferated for about 6 days, similar to Dhiman's model. It is clear that the MCF-7 blocks cannot be kept alive for long, and that the culture conditions are not suitable for preserving the entire tissue mass. Wang claims that inoculating 2D and 3D samples with MCF-7 cells yielded similar MTT results (day 4 0.35:0.3 and day 7 0.4:0.45) [14]. The experimental cultures were 0.3:0.3 and 0.4:0.5 on days 4 and 7. Thus, the static 3D model of MCF-7 cells was quite similar to the static 2D model.

Cell mass size and morphology in static culture

Both cell masses were spherical after 24 and 96 h of culture. Although MCF-7 cells and fibroblasts commonly stick to the surface of cultivated tissue and seem stellate, the cell masses in the experiment were spherical (**Figure 5**). It was evident, after 96 h of cultivation in both quantity and shape, the size of the 2-cell samples increased continuously, and the number of blocks increased dramatically (**Table 1**). The morphology of MCF-7 cell masses is round, bright, and clear. In the 1-cell sample, the cell mass appears fractured, the dark mass may be due to cell death, and the number of blocks is substantially less (**Figure 5**).

Table 1 The size and the number of cell mass in GA scaffold.

Date	One-cell sample (MCF-7)			Two-cell sample (MCF-7 and fibroblast)		
	Size (µm)	Quantity	Median (µm)	Size (µm)	Quantity	Median (µm)
4	43.18 - 124.27	17	90.99	41.56 - 129.17	20	76.04
6	48.83 - 143.57	23	97.47	56.62 - 186.16	19	93.31
8	69.01 - 141.16	16	107.67	74.81 - 143.61	21	116.41
10	53.52 - 147.79	20	81.40	72.51 - 190.94	10	146.49
12	31.05 - 117.50	6	51.47	65.39 - 175.95	13	106.14

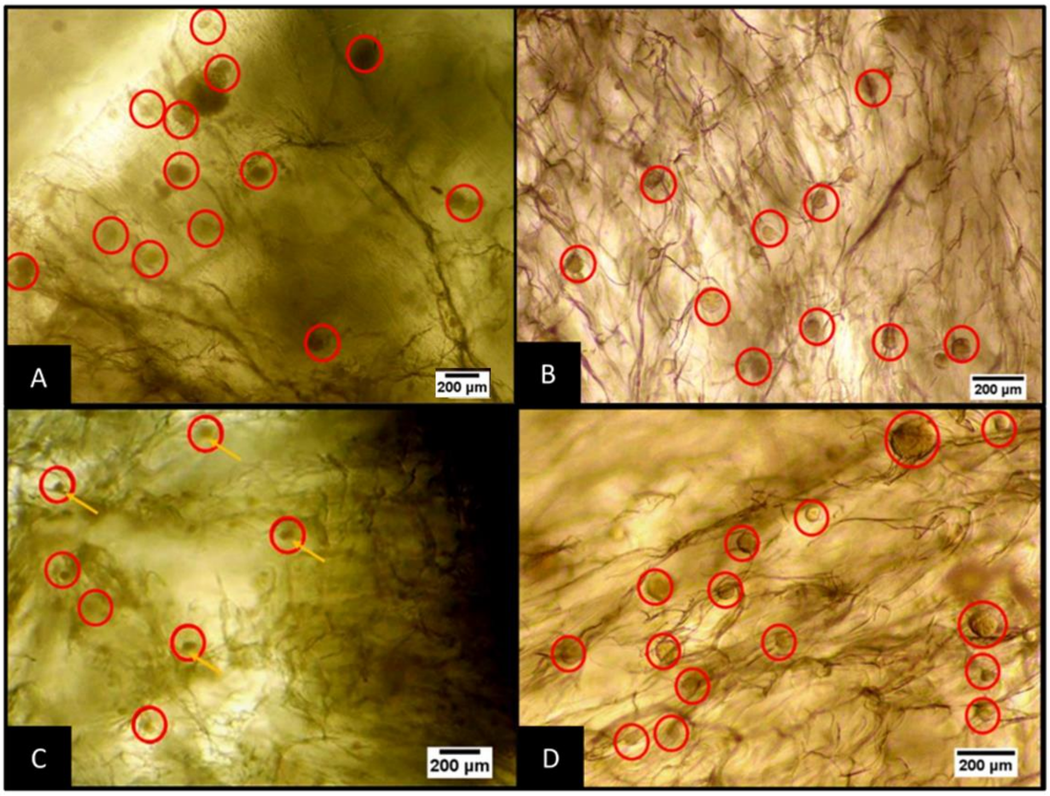

Figure 5 The MCF-7 cells in gelatin-alginate scaffold on inverted digital microscope A. One-cell sample after 24 h, B. Two-cell sample after 24 h, C. One-cell sample after 96 h, D. Two-cell sample after 96 h. **The red circles** show the cells in the GA scaffold.

Histological morphology in static culture

When MCF-7 was cultured for 7 days (**Figures 6**), the cells in the mass started to die off, the nucleus had a lot of white space, the nuclei disappeared, and some cells couldn't see the nucleus any longer. The mass was also broken up, and there were lots of floaty cells in it. In the combined sample, the cells had dark nuclei, but the nuclear membrane and nucleus were visible. Cells and aggregates had varying sizes and characteristics. In contrast to Xiong's cell mass, the structure of this mass has a greater degree of stability [15]. The reason can be that breast cancer cells adhere to 1 another thanks in part to human fibroblasts.

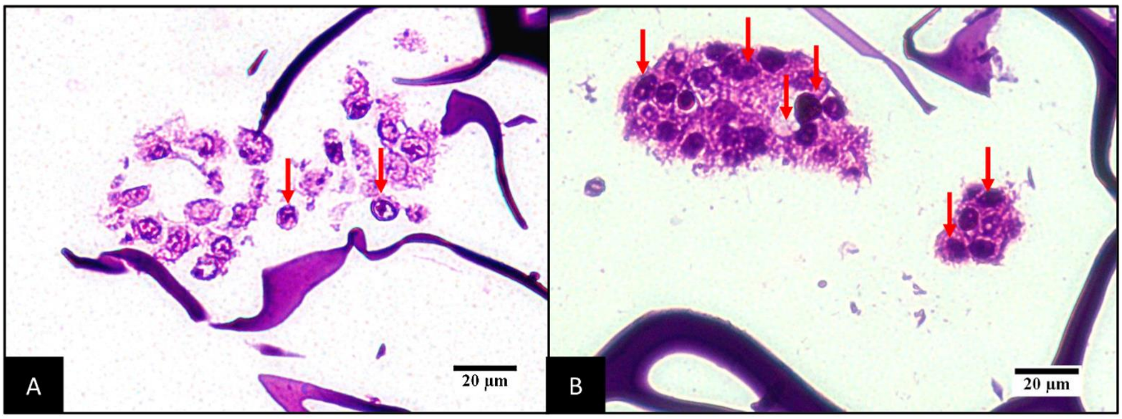

Figure 6 The H&E of MCF-7 cell masses in gelatin-alginate scaffold (A. One-cell sample after 7 days, B. Two-cell sample after 7 days). **The red arrow** shows the cell mass in the GA scaffold.

After 7 days of static culture, the usage of fibroblasts provided good support for the survival of cancer cells. Based on the results, we will use 2-cell sample in combination for future tests. The experiments were set up as follows in light of these findings. First, fibroblasts were introduced about 7 days before dynamic culture. Then, MCF-7 was seeded about 4 days before dynamic culture.

Evaluation of the proliferative capacity of the cell mass in a dynamic culture

When compared to a static culture, the rate of cell multiplication in an agitation culture in **Figure 7** was nearly constant (thrive up to day 4). The block's maintenance phase lasts for 12 days, as opposed to 2 days for the static culture, and the MTT figure stays between 0.7 and 0.9 throughout that time. However, the use of a bioreactor is more effective at promoting cell growth in the bulk compared to the other methods. Cell proliferation in the bioreactor progressively rose until day 8 then increased sharply, get a peak at day 16 and declined rapidly until day 24, and remained stable until day 28. The bioreactor samples had an MTT significantly figural difference between agitation and bioreactor with 0.7 and 1.7, respectively. There were no indicators of reduction in the cell mass between day 24 and day 28, indicating that the cell mass was continuing to increase without entering a decline phase. In a similar vein, Zheng (2012) did a shake culture of MCF-7 in collagen hydrogel-alginate in order to examine cell growth. During the 4-day culture period, cells grew at a rapid rate. The MTT assay has confirmed this finding. A sample of fibroblasts and proper shaking conditions may have contributed to the long-term viability of the sample in the Zheng experiment, which deteriorated swiftly after that [16].

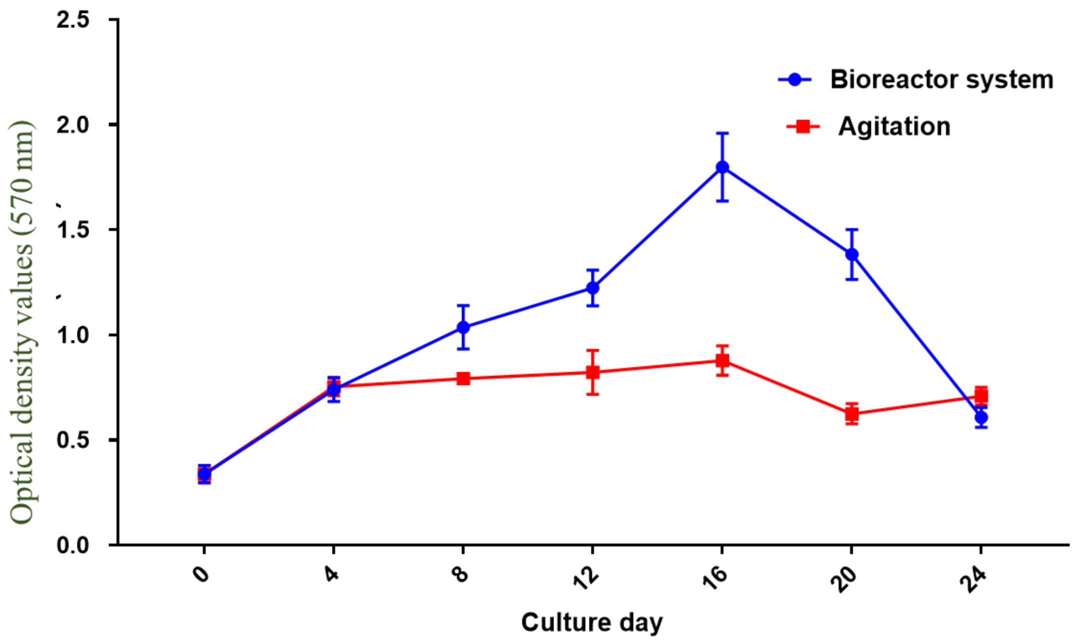

Figure 7 The proliferative rate of cells in GA scaffold in 2 dynamic culture methods.

Cell mass morphology in a dynamic culture

Injured cells can revert to their normal state if cultured under stable and controllable conditions of fluid flow speed, oxygen concentration, and nutrient content. For the mass to grow, the bioreactor's culture conditions must be met. One of the advantages of these masses is that they are more uniform and larger than static cultures. A steady stream of brightly colored blocks appeared on day 14, resembling the day 0 pattern depicted in **Figure 8**. There was a considerable drop in block count by day 21, and black blocks showed signs of cell death and disintegration, losing their round shape. It is possible that the setting is no longer suited.

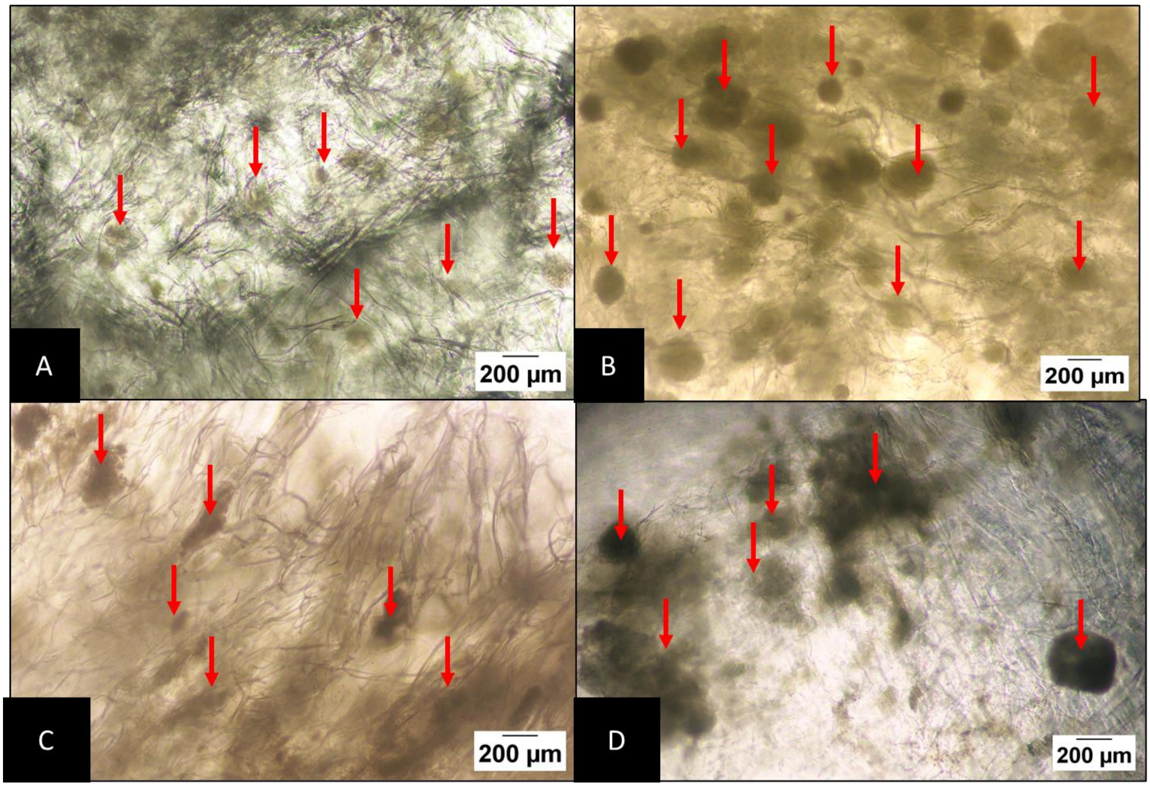

Figure 8 The morphology of mass in GA in bioreactor on the Inverted Digital Microscope at the observative time (A. day 7, B. day 14, C. day 21, D. day 28). The red arrow shows the masses in the gelatin-alginate scaffold.

Cell mass histological morphology in the dynamic culture

In **Figure 9**, the cell mass structure of the 7-day bioreactor was the same as the static culture, which contained numerous dead cells. Biomass and mass structure increased significantly when the culture conditions were changed. However, necrosis has caused a few big holes in the dense mass. However, masses began to rebound and grow nicely by day 14, primarily taking the form of lengthy bands. When the drooping mass is more developed than the sphere on day 7, this can be recognized. Having a banded structure reduces the lack of oxygen and nutrients in the middle of the mass, allowing the mass to expand more effectively. Dissociation of cells via apoptosis creates much more stable masses. A decline in cell proliferation and mass rupture began on day 21, a sign that the culture conditions were no longer amenable to mass preservation. Nuclei are still vividly colored, and the morphology is diversified; more conspicuous are the brightly colored fibroblasts in the last blocks. As before, the 28th block is thriving and expanding once more. The fibroblasts within the bulk darken in color as the mass becomes denser. Wang's work defined the MCF-7 cell mass as spherical and tightly packed [17]. This matches the cell mass morphology on the Gelatin-Alginate framework. The 2-cell sample treatments had lighter colored blocks bordered by a thin layer. Both samples had a similar block count. Formation of cell clumps in the scaffold with a consistent round spherical form, significant proliferation of MCF-7 cells on the GA scaffold surface. Cells extend to aggregate, forming a multilayer structure. Chen (2012) mentioned this [18]. There are many projecting cells on the tumor formation. Based on the morphological alterations and rearrangement of the cytoskeleton, Amaral (2010) attributes this to tubular breast cell structure [3]. MCF-7 and proliferating fibroblasts also doubled. Because of the early signals of programmed cell death with fading nuclei and holes in the mass, a change from shake culture or bioreactor is required to restore these building blocks to their original state. To regenerate, a cell must be placed in an environment where the damage is not too severe, yet necrosis and death will occur if the damage is acute and severe. There is no time to waste. As a result, the mass is more stable before it is fed into bioreactors, where the freshly produced blocks are more easily ripped off if supplied too early with a high shaking speed unit.

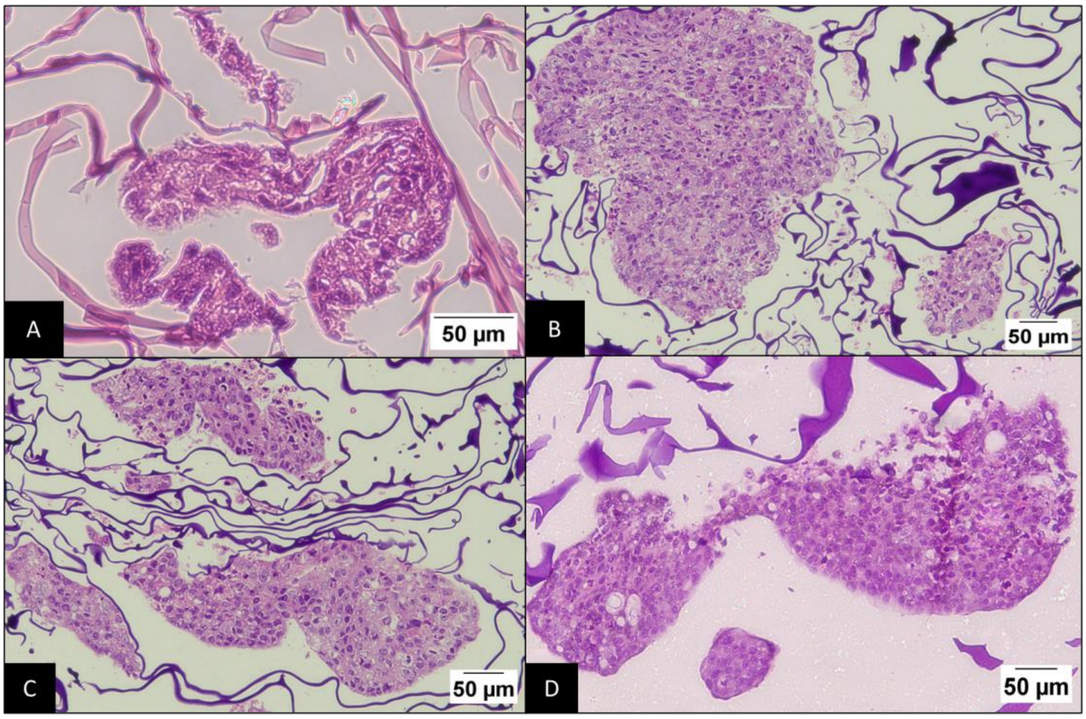

Figure 9 The histological morphology of mass in GA in the dynamic culture at the observative time (A day 7, B. day 14, C. day 21, D. day 28). The orange arrows show the cells in the mass.

Histological images show the growth of cells in the gelatin-alginate scaffold, but it is difficult to distinguish the cell type, number, and quality within the scope of the experiment. Understanding the interconnectedness of the cells, they require a wide range of resources and instruments. Furthermore, porosity, which includes pore size and structure, is critical for improving the cell seeding procedure. A typical manually cast scaffold, on the other hand, revealed an unstructured porous structure. As a result, 3-dimensional printing technology can produce a porous structure that is more organised [19].

Conclusions

The MCF-7 cells were incubated in Gelatin-Alginate for 20 min in the cell incubator, resulting in a stable cell-scaffold complex with even cell distribution. The efficiency rate of employing a bioreactor to culture MCF-7 cells with the support of fibroblasts at 37 °C and 5 % CO_2 is superior to traditional procedures such as static culture or agitation, according to the results. The structure of tumor mass has many identical points to that in a living organism. Furthermore, the tumor's growth is stable and has been for 28 days. As a result, the current work could be a big step forward in the development of the optimal scaffold for *in vitro* cancer research and therapeutic efficacy evaluation.

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