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NEURONAL TISSUE MODELLING WITH 3D BIOPRINTING

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ABSTRACT

In this study, SH-SY5Y neuroblastoma cells were bioprinted using a GelMA/HAMA bioink, and the formation of spheroids was observed to initiate as early as day 3. This observation is significant as it indicates that the specific composition of the bioink can have a profound impact on cellular behavior and the spheroid formation process. Comparatively, in earlier studies utilizing an alginate/gelatin-based bioink, spheroid formation only became noticeable around day 5, suggesting that the GelMA/HAMA bioink provides a more conducive environment for earlier cellular aggregation and organization. The results of this study highlight the critical role of bioink formulation in influencing the biological responses of cells within a three-dimensional (3D) microenvironment. The quicker spheroid formation observed with the GelMA/HAMA bioink may be attributed to its biochemical properties, such as enhanced cell adhesion, improved mechanical strength, and the ability to mimic the extracellular matrix. These properties provide an optimized platform for cell-cell and cell-matrix interactions, promoting accelerated tissue-like structures. This research underscores the importance of systematically investigating the effects of bioink compositions on cellular biology. Understanding the interplay between bioink components and cellular behavior is essential for improving the design and application of bioprinting technologies. Such insights are invaluable for advancing tissue engineering, regenerative medicine, and the development of disease models. Ultimately, the findings pave the way for creating more precise and effective biofabrication strategies, emphasizing the need for tailored bioink formulations that support specific biological outcomes in various applications.

Keywords: Bioprinting, Spheroid, Neuronal Tissue, GelMA/HAMA

1. INTRODUCTION

The discovery of three-dimensional (3D) printing has generated worldwide acclaim and led to significant industrial development. Although the idea of the emergence of 3D printers was seen as serving the industry, it did not take long to integrate into the health field. In such major developments, 3D printing technology has enabled the development of new devices, especially artificial tissues, and the development of prostheses [1]. In the 3D printing mechanism, the logic of printing this object together with its layers by determining the structural and functional properties of any object supported by a computer lies. 3D printing is even known as additive manufacturing. Today, 3D printer technology, which is especially used in the printing of prostheses and its use in this field is increasing day by day, has been used to create special implants and surgical guides using titanium, polyether ether ketone, hydroxyapatite and various biomaterials [2]. Tissues with a relatively less complex structure, such as skin and cartilage tissue, can be commercially produced and implanted with this technology, while this application cannot yet be successfully performed in tissues with a more complex structure. This has led to 3D bioprinting becoming a tool that makes it possible to produce both tissue structure and artificial organs in transplantation and other biomedical applications [3,4]. 3D Bioprinting carries the main idea of creating conditions similar to the environment of a real organism by analyzing structures whose anatomical

structure is known and can fall within the field of this technology. Here, cell-cell and cell-matrix interactions have a great advantage both in the printing process of the tissue or organ and in the ability to model it [5]. At this point, the process of tissue printing enters the field of tissue engineering. The feasibility of 3D bioprinters and organ transplantation should be carried out within the scope of the principles and rules of tissue engineering. In this context, the main components of tissue engineering consist of tissue scaffold, cells, and biosignaling molecules [6]. The material formed by the combination of natural or synthetic polymers used to form cells and cell scaffold material, living tissue, and organs desired to be obtained is called bioink [7]. Bio-ink can exhibit various structural and chemical properties including porosity, permeability, and mechanical strength, enabling the creation of a complete tissue environment [8]. Although scaffolds have been used in tissue engineering for a long time, there are still many limitations in the creation of tissues and organs. The most important limitation of scaffolds is that they do not fully mimic the natural extracellular matrix (ECM) function [9,10]. Cells tend to form tissue in favorable environments and conditions. In this case, when the cells are cultured in a suitable bioink in 3D culture systems, the cells form a spherical structure by adhering to each other rather than to the surface. This is called a spheroid [11].

1.1. Bioprinting Technologies

There are 3 methods of printers used in 3D bioprinting technology.

1-Extrusion Bioprinting [12],
2-Droplet/İnkjet Bioprinting [13],
3-Laser-Based Bioprinting [14],
4-Stereolithographic-Based 3D Bioprinting [15].

These methods are selected according to criteria that include many factors, including the characteristics of the tissue or organ to be printed, and the characteristics of the bioink to be used. All these methods have some advantages and disadvantages.

- **Extrusion Bioprinting**

The extrusion-based bioprinting technique is a technique that has the principle of working with the help of pressure or a mechanical piston to obtain the 3D shape. Here, the bioink is distributed in a temperature-controlled manner [16]. Extrusion printing uses viscous solutions, natural or synthetic polymers, and cell suspensions to print the printed tissue, this method is also called direct ink writing (DIW). This printing technique is the most commonly used method [17]. Therefore, it has many advantages. These advantages are;

1- Simple structure.
2-Speed can be controlled,
3- It makes it possible to print a wide range of biomaterials,
4- Having the ability to print more than one material,
5- It can print cells of various densities,

The biggest problem with such an advantageous extrusion bioprinter is that the cell viability rate is also lower than other types of printers [18]. In addition to this problem, the viscosity of the material to be used must also be appropriate due to its mechanism [19]. There are 4 stages in extrusion bioprinting.

I- *Stage I:* application of force to initiate and maintain extrusion,
II-*Stage II:* extrusion and filament formation,
III-*Stage III:* Top-down 3D deposition,
IV-*Stage IV:* cross-linking of biosupported structures to ensure mechanical integrity [20].

The studies obtained aortic valves [21], liver [22], heart and adipose tissue [23], bone and cartilage [24], skin [25], muscle [26], and vascular network using extrusion-based bioprinters [27].

- **Droplet/İnkjet Bioprinting**

Droplet/Inkjet bioprinting uses thermal, piezoelectric, and electrostatic tips to print bio-ink in a controlled manner using atmospheric pressure and fluid mechanics to form droplets. It is very important to control the properties such as spray surface tension and viscosity of the bioink in different tip types [28,29]. This bioprinter enables much faster printing compared to the extrusion method [30]. Droplet/Inkjet bioprinting has many advantages.

These advantages are;
1- Fast printing capability,
2-High resolution,

3- Low cost,
4-The ability to successfully print low-viscosity biomaterials,
5-Simple structure
6- Suitable for the use of many biomaterials such as alginate, gelatin, collagen, fibrin etc [28].

In addition to these advantages, inkjet bioprinting, which is frequently preferred in artificial tissue printing, has some disadvantages. The most important problem is that cell density can remain limited [31]. The inkjet bioprinter was used to print microvascular networks [32], cartilage tissue [33], and skin [34], and the tissues were successfully obtained. Although this technology was introduced in the early 2000s, the fact that it is still more limited in use shows that the studies to be carried out on this subject are very important.

- **Laser-Based Bioprinting**

The working principle of this technology, which was introduced in 1999, consists of several layers. Accordingly, laser bioprinters apply a pulsed laser beam onto a slide called a donor, which is coated with a layer that absorbs the laser energy and then vaporizes the material. At that time, a high-pressure bubble is created towards the acceptor substrate placed under the donor strip [35,36]. Some bioprinter types researched in laser-based bioprinter technology are; using matrix-assisted pulsed laser evaporation-direct writing (MAPLE-DW) [37], Absorbing Film Assisted Laser-Induced Forward Transfer (AFA-LIFT) or biological laser processing (BioLP), and Laser-Induced Backward Transfer (LIST) [38] and using continuous wave (CW) lasers, such as Laser Guided Direct Writing (LGDW) [39], bioprinters [40]. There are some advantages and disadvantages in this method as in the others.

These advantages are;
1-High cell viability,
2-High resolution,
3-Making it possible to print biomaterials in solid or liquid phase,
4- Without a nozzle. In this case, it is directly related to viscosity, and problems such as clogging in other printers are not experienced here.

In addition to these advantages, the high cost and the occurrence of tissue damage due to laser light are still seen as the biggest disadvantages [41]. A major step forward has been made in the printing of cells using laser-assisted bioprinting of tissues such as skin [42], and bone [43]. However, laser-assisted bioprinters have many challenges that need to be solved.

- **Stereolithographic-Based 3D Bioprinting**

The stereolithographic bioprinting method is the working principle of creating a layered structure of the design by adding materials by reflecting light planarly on the photosensitive heat-curable bio-ink. Here it depends on the height rather than the complexity of the texture [15,44]. Stereolithographic bioprinting is divided into 2 classes. The first is the single-photon method and the second is the multi-photon method. Traditionally, the single photon method is used. This method can be subdivided into 4 subdivisions:

1) Visible radiation systems,
2) Traditional stereolithography,
3) IR stereolithography systems,
4) Stereo-thermal lithography systems. Light projection systems can be applied directly with laser writing, and mask projection systems can also be used physically or digitally [45,46].

Several major problems characterize stereolithographic-based 3D Bioprinting technology. This limits the development of 3D printing and the full potential of products for use in healthcare fields such as dentistry and, more generally, the printing of fully functional parts, although temporary restorations, models, and prototypes can still be printed [47]. Innovative engineering approaches that can solve problems with this technology are also needed.

1.2. Current Developments in Bioprinting

It is seen as one of the most important steps to be taken in the field of health, both to meet the ever-increasing need for organ transplantation and to develop tissues or organs that can be implanted instead of the damaged tissue formed after degeneration. In this context, the production of artificial tissues and organs and thus the use of bioprinters has become a new breath of fresh air in the field. 3D bioprinting has an important place in tissue engineering

due to its many advantages, both in terms of controllable morphology and high resolution. Many tissue types, including bone, cartilage, skin, vascular system, heart, and neuronal tissues, have been produced using various bioprinting approaches, and skin and cartilage tissues have even been commercialized. In light of this information, the idea that organ printing can be realized with the help of 3D bioprinting technology in some diseases such as kidney and liver, where survival is possible with organ transplantation, has guided the studies in this field. However, it still seems to be beyond the limits of the printed organ [48,49]. The successfully printed tissues and organs are summarised in Table 1.

Table 1. Various 3D bioprinted tissues

3D Bioprinted Tissues	Bioprinter technology	Bioinks	Cell Viability Rate After Printing	Ref.
Cartilage	Inkjet bioprinter	Polyethylene glycol (PEG)	approximately 90%	[50]
	Extrusion bioprinting	Alginate Sulfate- Nanocellulose	approximately 85%	[51]
	Stereolithographic-Based bioprinting	Methacrylate Gelatin (GelMA) And Methacrylated Hyaluronic Acid (HAMA)	approximately 95%	[52]
Bone	Inkjet bioprinter	PEGDMA, GelMA,	approximately 85%	[53]
	Extrusion bioprinting	Alginate-polyvinyl alcohol (PVA)-hydroxyapatite (HA)	average viability values of 95.6% and 77.5%,	[54]
Liver	Extrusion Bioprinting	Polycaprolactone (PCL)	approximately 85%	[55]
Cardiac Tissue	Extrusion Bioprinting	Fibrinogen, Aprotinin, Hyaluronic Acid	-	[56]
Neuronal	Inkjet bioprinter	Human dermal fibroblasts, NG108-15 neuronal cells, Schwann cells	cell viabilities of >86% and >90%	[57]
Blood Vessels	Laser-based	Matrigel, agarose	-	[58]
	Extrusion Bioprinting	GelMA	-	[59]
	Inkjet bioprinter	Alginate	-	[60]

Printing of neuronal nerve tissue with 3D bioprinting technique and in vitro neuronal tissue formation will help to elucidate the unknown mechanisms in this structure. Studies have also shown that the continuity of cellular functions is ensured by printing neuronal cell lines with the help of a bioprinter and that organism-like physiological responses occur. Beyond all these systems, the nervous system contains a wide variety of cell types and constitutes a complex structure.

There are a variety of neurons and glia cells distributed in different parts of the nervous system. The brain, a member of the central nervous system, is thought to contain approximately 86 billion neurons and 85 billion glia and other cell types. This shows how complex it is as a structure [61].

In this study, it was aimed to create brain-like spheroids of SH-SY5Y cell line, which is a neuroblastoma cell line, using Extrusion Bioprinting and to analyse the tissue structure with Fujı programme and Waikato Environment for Knowledge Analysis.

2. MATERIAL METHOD

In this study, the SH-SY5Y cell line was prepared in commercially available GelMA/HAMA, printed on well plates in a 3D bioprinter and left for incubation and spheroids were observed.

2.1. SH-SY5Y Cell Line Cultivated

For 3D culture, the SH-SY5Y cell line was first cultured. SH-SY5Y cells were cultured in DMEM-F12 (Dulbecco's Modified Eagle's Medium) medium containing 10% Fetal Bovine serum, L-Glutamine, non-essential

amino acids, sodium pyruvate and sodium bicarbonate at 37^0C in an incubator containing 5% CO_2. The medium was changed every two days. Cultivation was continued until the cells reached sufficient density. The cultured SH-SY5Y cell line was washed twice with 5 mL PBS by removing the medium. After washing, 5 mL 0.25% trypsin and 2.21 mM EDTA 4Na were added to the flask and incubated at 37 °C for 5 minutes. After incubation, 10 mL DMEM-F12 medium with 10% FBS was added to the flask and the suspended mixture was transferred to a centrifuge tube. It was centrifuged at 100xg for 5 minutes. After centrifugation, the supernatant was removed. 1 mL fresh medium was added to the remaining pellet [62].

2.2. Creating the Bioink

Commercially available lyophilised GelMA and HAMA powders were obtained. Lyophilised GelMA powder was dissolved in DMEM at 80 °C and 12.5% gelatin solution was collected. Lyophilised HAMA powder was dissolved in DMEM at 37 °C and a 2% hyaluronic acid solution was collected. Similarly, both GelMA and HAMA powders were dissolved in DMEM and a 10%/1% GelMA/HAMA solution was collected. All solutions were sterilised after filtration through a 0.22 µm strainer (Falcon). The solution was exposed to 405 nm blue light for 30 seconds to form the hydrogel structure [63].

2.3. Bioink Characterization

The prepared GelMA/HAMA was characterized by emission scanning electron microscopy (SEM) and imaging and water retention capacity.

After crosslinking, 12.5% GelMA/HAMA hydrogel structures were frozen in liquid nitrogen, lyophilized, and sputter-coated with Au-Pd (2 nm). The structures were examined by field emission scanning electron microscopy.

To evaluate the swelling (water retention) capacity of the 12.5% GelMA/HAMA hydrogel, a sample gel was incubated in medium for one week. On day 0 and day 7, the weight of the hydrogel was measured on a precision balance to understand the swelling capacity.

$$SC: \frac{Wf-Wi}{Wi} X100 \quad (1)$$

Wf: final weight of the hydrogel
Wi: initial weight of the hydrogel

2.4. Printing Neuronal Cells With a 3D Bioprinter

The neuronal cells to be printed with the bioprinter were washed twice with PBS after being removed from the medium. The cells were then incubated with trypsin, removed, centrifuged to remove the supernatant and resuspended in DMEM-F12 medium containing 10% FBS. Cell counting was performed using a Thoma slide and the cell concentration was determined as 1.5 x 10^6 cells/mL. The prepared bioink hydrogels were heated to 37℃ and cultured SH-SY5Y cells were added into it. To prepare cell-loaded hydrogels, a 5:1 hydrogel:cell mixture was prepared by pipetting slowly to avoid the formation of bubbles. The cell-loaded hydrogels were then taken into 3 ml pneumatic tubes compatible with the bioprinter and incubated in 37℃ 5% CO_2 environment until the printing stage. It is shown in Figure 1.

Figure 1: A: Cell-loaded hydrogels B: Bioprinter syring

Cell-loaded hydrogels were fabricated using the Axo A6 Bioprinting System, an extrusion-based three-dimensional bioprinting system. The neuronal tissue was first realized with the computer-aided PrusoSlicer software. The total size of the constructs was designed as 23 mm x 23 mm x 2 mm. The bioprinted constructs were extruded as 3 circular layers for each well. The cell-containing hydrogel was placed in bioprinter syringes and loaded into the bioprinter for sterile media printing. The bioprinting program is shown in Figure 2.

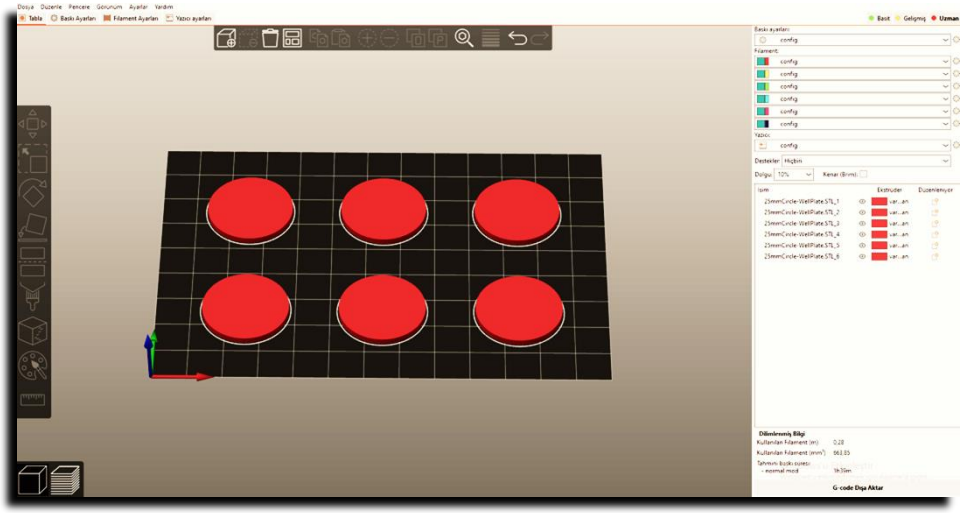

Figure 2: Designing the printing of cellular hydrogel on 6-well plate

Then, 6 well plates were placed on the bioprinter table, calibration and orientation of the printer were performed with Repetier-Host software. By optimising the printing parameters, cellular hydrogel with a final height of 1 mm was printed at room temperature and at a speed of 100. After printing, the medium was carefully removed from the cells every two days and replaced with sterile and fresh medium. The culture continued for 5 days and the cells were examined microscopically.

2.5. Live & Dead Test

Fluorescence staining was performed for viability analysis of cells printed using Live and Dead Cell Assay (ab115347). At the end of all treatments, the media in all wells were carefully removed. Ca+2 was captured with PBS without Mg+2, followed by the addition of Live & Dead test solution containing 2 µM Calsein AM and 4 µM EthD-1 and incubated for 45 minutes at room temperature. At the end of the incubation, they were washed again with Ca+2, Mg+2-free PBS and microscopic observation was performed under a fluorescence microscope (Zeiss, Germany) [62].

3. RESULT

SH-SY5Y cell line, which is known as dopaminergic neuroblastoma, is frequently used in in vitro disease modelling of many diseases in the literature. In our study, we cultured SH-SY5Y cells with DMEM-F12 (Figure 3).

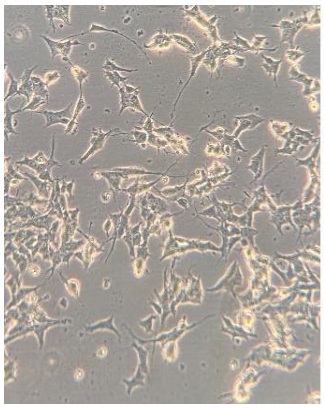

Figure 3: SH-SY5Y cell line

The prepared GelMA/HAMA biosurfactant was then characterised by SEM and water retention capacity determination analyses.

The obtained GelMA/HAMA hydrogel was found to have a gel form and viscosity that can be used as a bioink in 3D cell culture. GelMA/HAMA hydrogel prepared with a diameter of 1 cm was syringed into the well plate and after cross-linking, DMEM-F12 medium containing 4 ml of 10% FBS was added. The water retention capacity was measured on day 0 and day 7 on a precision balance and the swelling capacity of the hydrogel was determined as a percentage according to the formula described above.

Table 2. Hydrogel weight

Wi	***Wf***
1,35g	2,18g

The hydrogels measured on day 0 and day 7 were 1.38g on day 0 and 2.68g on day 7.

Accordingly, 61.48% swelling was observed on the 7th day.

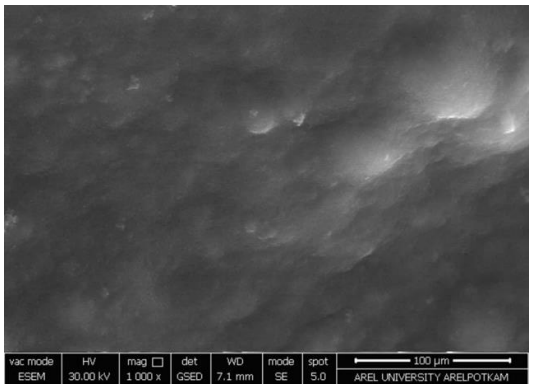

Figure 4: SEM imaging of the hydrogel

SEM imaging of the hydrogel also showed that it has a smooth structure

The SEM image of the bioink is shown in Figure 4.

The characterised bioink was sterilised and mixed with SH-SY5Y cell line and medium. It was loaded into the bioprinter syringe and 6-well plate was inoculated. After inoculation, observed. 3D cell culture analysis continued until day 5 and images of the cells were taken.

3D cell culture analysis continued until day 5 and images of the cells were taken. At the end of the 5th day, spheroids were observed under fluorescence microscope with live&dead staining and images were taken. The images were then uploaded to the Fiji programme and the 3D size graph was draw.

medium was added to each well and cultured by incubation in the incubator. Wells were checked by light microscopy. As seen in Figure 5 and 6, daily spheroid formation was

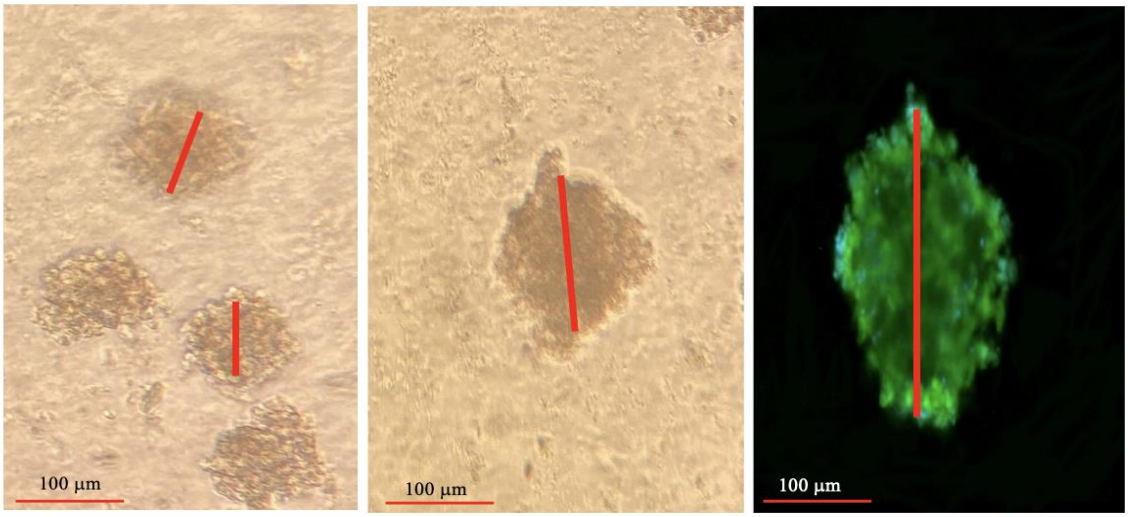

Figure 5: Spheroidal form in neuronal cell line. A: Cell image in 2D culture, B: Spheroid image in 3D culture system, C: Fluorescence microscope image in spheroid Live&Dead assay

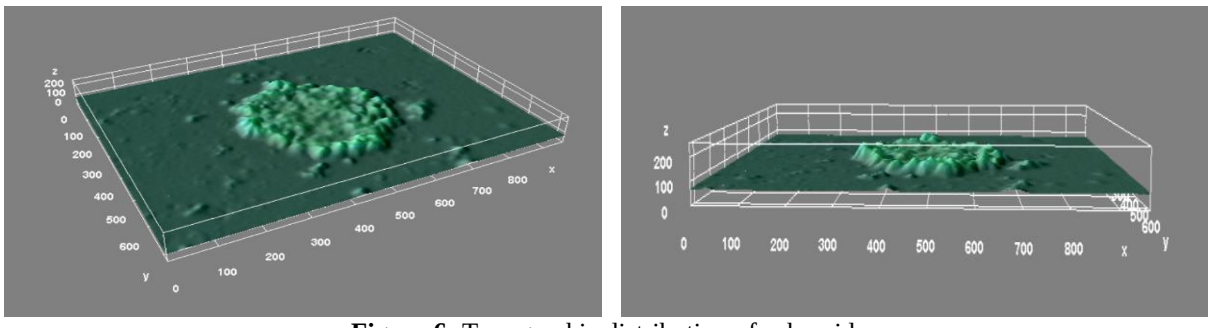

Figure 6: Topographic distribution of spheroid.

4. CONCLUSIONS

In our study, SH-SY5Y cells were printed with GelMA/HAMA bioink with a bioprinter and spheroid formation was observed to start on day 3. In our previous studies, an alginate/gelatin-based bioink was prepared and

it was determined that spheroid formation started to become evident after 5 days.

The products obtained or to be obtained thanks to 3D Bioprinting technology, as part of additive manufacturing, hold great promise in the field of tissue and organ regeneration. This technology, which can eliminate the ongoing transplantation problems in the clinic, aims to increase both the chance of survival and quality of life for many patients. Another advantage of this technology is that it can be said to be advantageous compared to autografting or allografting, considering the stress of autologous grafts on the patient and acute deficiencies in allograft donors. In terms of the working principle of 3D bioprinting, it is considered one of the most important technologies of today, as it minimizes the risk of immunological graft rejection and problems related to a donor shortage, and offers a unique opportunity in this field. In addition, in this technology, tissue or organ imprinting can also be possible with cells taken from the patient. In this case, it can completely overcome the problems that may arise immunologically. Of course, this situation is not only immunological. In addition, it can eliminate aesthetic concerns. Although progress has been made at a tremendous pace, especially in recent years, there are still many problems to be solved. The most important of these problems is the biocompatibility of the printed structure and its integration with the body. Considering the range of bioprinters currently in use, another element that needs to be researched and developed is bio-inks. However, even if all these factors are solved, standardization studies and quality control studies related to the field need to be completed. Considering the development process, we think that this quite new field has a lot of work ahead of it and has the power to solve many problems. In short, we foresee that 3D bioprinting will play a very important role in producing functional tissues and organs by modeling them not only for transplantation but also for use in drug screening procedures and physiological studies.

Bioprinters, which have a multidisciplinary field of study, can overcome all difficulties, and we anticipate that efficient results can be obtained at the end of joint research in various fields such as engineering, biology, chemistry, computer, and medicine.

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