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Corresponding Author	Family Name	**Chapa-González**
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	Organization	Instituto de Ingeniería y Tecnología de la Universidad Autónoma de Ciudad Juárez
	Address	32310, Ciudad Juárez, CH, Mexico
	Email	christian.chapa@uacj.mx
	ORCID	http://orcid.org/0000-0003-1760-6116
Author	Family Name	**Valenzuela-Reyes**
	Particle	
	Given Name	**Marcos Bryan**
	Prefix	
	Suffix	
	Role	
	Division	
	Organization	Instituto de Ingeniería y Tecnología de la Universidad Autónoma de Ciudad Juárez
	Address	32310, Ciudad Juárez, CH, Mexico
	Email	
	ORCID	http://orcid.org/0000-0002-8226-6931
Author	Family Name	**Alemán-Miranda**
	Particle	
	Given Name	**Lizbeth Lucero**
	Prefix	
	Suffix	
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	Organization	Instituto de Ingeniería y Tecnología de la Universidad Autónoma de Ciudad Juárez
	Address	32310, Ciudad Juárez, CH, Mexico
	Email	

Author	Family Name	**Valencia-Gómez**
	Particle	
	Given Name	**Laura Elizabeth**
	Prefix	
	Suffix	
	Role	
	Division	
	Organization	Instituto de Ingeniería y Tecnología de la Universidad Autónoma de Ciudad Juárez
	Address	32310, Ciudad Juárez, CH, Mexico
	Email	
Author	Family Name	**Botello-Arredondo**
	Particle	
	Given Name	**Adeodato Israel**
	Prefix	
	Suffix	
	Role	
	Division	
	Organization	Instituto de Ingeniería y Tecnología de la Universidad Autónoma de Ciudad Juárez
	Address	32310, Ciudad Juárez, CH, Mexico
	Email	
Author	Family Name	**Zúñiga-Aguilar**
	Particle	
	Given Name	**Esmeralda Saraí**
	Prefix	
	Suffix	
	Role	
	Division	
	Organization	Instituto de Ingeniería y Tecnología de la Universidad Autónoma de Ciudad Juárez
	Address	32310, Ciudad Juárez, CH, Mexico
	Email	
	ORCID	http://orcid.org/0000-0002-6174-0729

Abstract

Tumor spheroids is a 3D culture of cancer cells. This type of cell culture is a great tool for the evaluation of novel nanomedicine systems and in other areas of biomedical engineering. The main advantage over monolayer cell cultures is the biomimetic microenvironment which is appropriate for recapitulating tumor complexity. However, current tumor spheroids obtention methods require sophisticated and expensive equipment and are time-consuming. It is possible to obtain these tumor spheroids by centrifugation of the suspended cancer cells in round-bottom tubes and using compaction agents, for example agarose, which is a polysaccharide well known for its function of forming gels. Herein, we developed a method for obtaining cancer spheroids varying the centrifugation time and the concentration of agarose. The variation in spheroid size was analyzed. No significant changes were observed in the morphology or in the initial size and growth of the spheroids; except in those obtained with the shortest centrifugation time. The cell viability of spheroids that showed growth as a function of incubation time was evaluated. Viability greater than 80% was presented, however, the cell viability does not grow when the size of the spheroidal tumor increases. This simple and effective method for obtaining in vitro tumors represents a tool to further studies in Nanomedicine systems or the development of new anticancer drugs.

Keywords: Cancer - Tumor spheroid - Cell viability

Formation of Tumor Spheroids by Spontaneous Cellular Aggregation in Incubation: Effect of Agarose as a Compaction Agent

Christian Chapa-González(✉), Marcos Bryan Valenzuela-Reyes, Lizbeth Lucero Alemán-Miranda, Laura Elizabeth Valencia-Gómez, Adeodato Israel Botello-Arredondo, and Esmeralda Saraí Zúñiga-Aguilar

Instituto de Ingeniería y Tecnología de la Universidad Autónoma de Ciudad Juárez, 32310 Ciudad Juárez, CH, Mexico
christian.chapa@uacj.mx

Abstract. Tumor spheroids is a 3D culture of cancer cells. This type of cell culture is a great tool for the evaluation of novel nanomedicine systems and in other areas of biomedical engineering. The main advantage over monolayer cell cultures is the biomimetic microenvironment which is appropriate for recapitulating tumor complexity. However, current tumor spheroids obtention methods require sophisticated and expensive equipment and are time-consuming. It is possible to obtain these tumor spheroids by centrifugation of the suspended cancer cells in round-bottom tubes and using compaction agents, for example agarose, which is a polysaccharide well known for its function of forming gels. Herein, we developed a method for obtaining cancer spheroids varying the centrifugation time and the concentration of agarose. The variation in spheroid size was analyzed. No significant changes were observed in the morphology or in the initial size and growth of the spheroids; except in those obtained with the shortest centrifugation time. The cell viability of spheroids that showed growth as a function of incubation time was evaluated. Viability greater than 80% was presented, however, the cell viability does not grow when the size of the spheroidal tumor increases. This simple and effective method for obtaining in vitro tumors represents a tool to further studies in Nanomedicine systems or the development of new anticancer drugs.

AQ1 AQ2 AQ3

Keywords: Cancer · Tumor spheroid · Cell viability

1 Introduction

Cell culture methods have spread prolifically within a century [1]. Cell culture has represented a powerful tool to progress on investigation cellular responses to drugs [2] and novel anticancer nanomedicine systems [3, 4]. In 2D culture systems, cells are grown as monolayers on a flat solid surface, missing cell interactions that are present in native organs or tumors. In contrast, 3D culture systems encourage cell aggregation and

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compaction which mimics tumors *in vivo*. As fields such as nanomedicine advance there is an increasing need to develop cell culture models to study the uptake of nanomaterials systems by tumor cells. Recently, it was shown that cancer spheroids are appropriate for studying the effect of nanoparticle physicochemical properties on the penetration and the uptake into tumors *in vitro* [5]. Cell spheroids offers the advantage of better imitating the complexities of tumors as compared to conventional monolayer culture systems [6]. However, multicellular spheroid fabrication methods employ sophisticated techniques such acoustofluidics [7], laser direct-write (LDW) bioprinting [8], microfluidic system [9, 10], and self-filling microwell arrays (SFMAs) [11] which are costly, and of intensive-labor, as well as time-consuming.

Mechanical agitation of suspended cells is a simple and effective technique to obtain cell spheroids. In this method the individualized cells are seeded in a culture medium, followed by centrifugation. The cells spontaneously aggregate themselves to form spheroids while remaining attached. Gentle agitation during incubation at 37 °C promotes the cells to remain anchored in the aggregate. However, to achieve the best cellular viability, growth, and homogeneity of spheroids formed it is still necessary to evaluate experimental variables such as time of centrifugation and the addition of molecules that allow the aggregation when obtaining tumor spheroids.

In the present study, we have developed a convenient methodology to assess the formation and growth of tumor spheroids. We have examined and compared the effects of the time of agitation and the addition of agarose on the viability as well as the compaction of cancer cells. From these several results, we aim to recognize the conditions that ease the formation of the tumor spheroids.

2 Materials and Methods

2.1 Spheroid Culture

Cervical cancer cells were grown in DMEM supplemented with 10% fetal bovine serum, and 100 U/ml penicillin at 37 °C in a humidified 5% CO_2 atmosphere. The medium was replaced every 3–4 days. The cells were detached using trypsin and counted in the Countess, Automated Cell Counter (Invitrogen) using trypan blue staining. Then, 1.5×10^4 viable cells were placed in six Eppendorf tubes containing cell culture medium with 20 µL of RPMI 1640 and 50 µl of agarose (1.5% w/v). Agarose was previously sterilized in a water bath. Other six tubes were prepared, but without containing agarose. Then three tubes containing agarose and three without agarose were centrifugated at 1500 rpm for 6 min while the other six tubes were centrifuged for 15 min. Then, the precipitate was incubated at 37 °C in a humidified 5% CO_2 atmosphere for 4 days. The medium (500 µL) was replaced with the fresh one every 24 h.

2.2 Morphology and Growth of the Cancer Spheroids

The spheroids obtained after the centrifugation of the cancer cells were kept in moderate agitation for 1 h and then transferred to a sterile culture plate. The size and shape of cancer spheroids were recorded by using an inverted light microscope. Spheroids

were imaged daily for 4 days by using an Axiocam ERc 55 of the Zeiss Vert A1-Ax10 microscope. The pictures were analyzed, and the diameter of spheroids was determined by measuring their cross-sectional area.

2.3 Cell Viability Assay

Spheroids were transferred from the tubes into wells of an uncoated 24-well plate. The medium surrounding the spheroids was removed out, and the spheroids were washed twice with PBS 1x, pH 7.4. The spheroid was dissociated with trypsin to obtain a suspension of cells. At the same time, 1.5×10^4, 3.0×10^4, 4.5×10^4, 6.0×10^4, and 7.5×10^4 cells were also moved to a 96-well plate to perform a calibration curve on them. MTT reagent was added to each well and the plate was incubated at 37 C, 5% CO_2 for 1 h. The absorbance of the plate was read at 540 nm by using a NanoDrop™ 2000 Spectrophotometer (Thermo Fisher Scientific).

3 Results and Discussion

Images of the aggregates were taken and then the average diameter was calculated (n = 12). In Fig. 1 it is observed representative tumor spheroids formed after two days of incubation. Some of the aggregates did not have a defined spheroidal shape, regardless of whether the culture medium contained agarose or not. This means that the addition of this polysaccharide does not modify the obtained morphology. However, the measurements were made in those aggregates with spheroidal morphology. It was also not observed that the centrifugation time favored the formation of multicellular aggregates with spheroidal morphology. Regardless of the centrifugation time, spheroidal and other irregular morphologies were observed in the cultures. The formation of aggregated tumor spheroids consists of four stages of formation which are compaction, segmentation, division and proliferation. The compaction stage occurs at the beginning on day 1, during this stage the initial compaction of the cancer cells occurs. In addition, on day 1, a high compaction of the cancer cells occurs that allows the formation of the spheroid structure.

We further characterized the growth of aggregates as a function of incubation time with the initial seeding density of 15 000 cells. It was expected that the size of spheroids was proportional to the initial cell numbers. However, a significant difference in the initial size and in the size growth of the tumor spheroids were noted in those obtained in culture medium lacking agarose with a centrifugation time of 6 min. In Fig. 2 it can be noted that the tumor spheroids tend to decrease in size depending on the incubation time, except for the case mentioned above. The spheroid diameter was approximately approaching 150 μm on day 1 with a low variation. While the initial average size of the spheroids in the highlighted method was 687 μm. Moreover, it was seen a growth of almost 3 times the average of the initial size while the average of the tumor spheroids diameter of the remaining methods decreased. It is possible that the agarose prevents the passage of nutrients and the aggregation of more cells, and this affects the observed size. Apparently, increasing the centrifugation time does not increase the initial average size of the aggregates, on the contrary it is reduced almost 4 times.

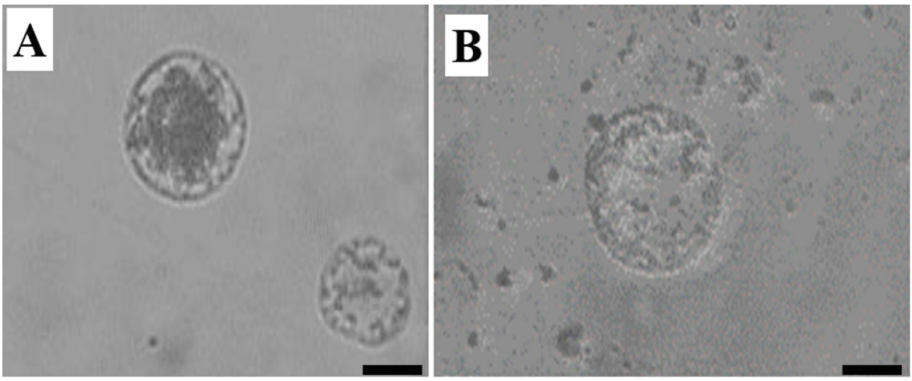

Fig. 1. Representative images were taken in the microscope (20X) where the tumor spheroids are observed (A) Tumor spheroid formed with cell media containing agarose, centrifuged 15 min at 1500 rpm. (B) Tumor spheroid formed with cell media without agarose, centrifuged 15 min at 1500 rpm. Culture days = 2. Scale bar = 100 μm

If it is considered that the physiological state of cells in a spheroid depends on its spheroid size, we must verify that the growth of the tumor spheroid is due to the aggregation of viable cells. The MTT assay was performed in order to obtain the cell viability of the multicellular spheroids (Fig. 3). Results showed that cells aggregates have a cell viability greater than 80%. A decrease in viability occurs in day 2 which can be attributed to the compaction stage. The stage of the segmentation takes place from the second day of incubation where the formation of the three specific zones of the cancer spheroids takes place: the zone of proliferation, the zone of senescence, and the necrotic zone. Cancer cells divide and proliferate with a low cell density which allows the formation of these zones which affects the cell viability as it can be seen.

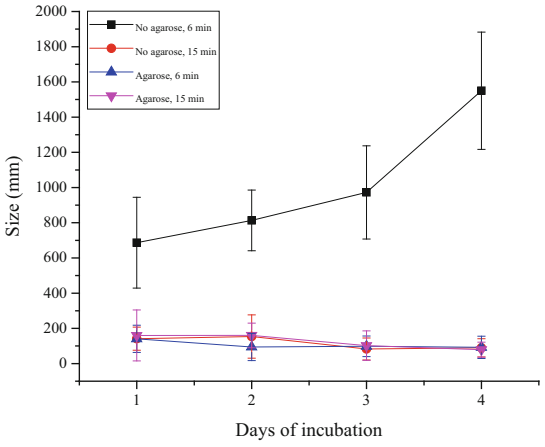

Fig. 2. Graph of variation of the average diameter of the tumor spheroids (n = 12) as a function of the incubation time.

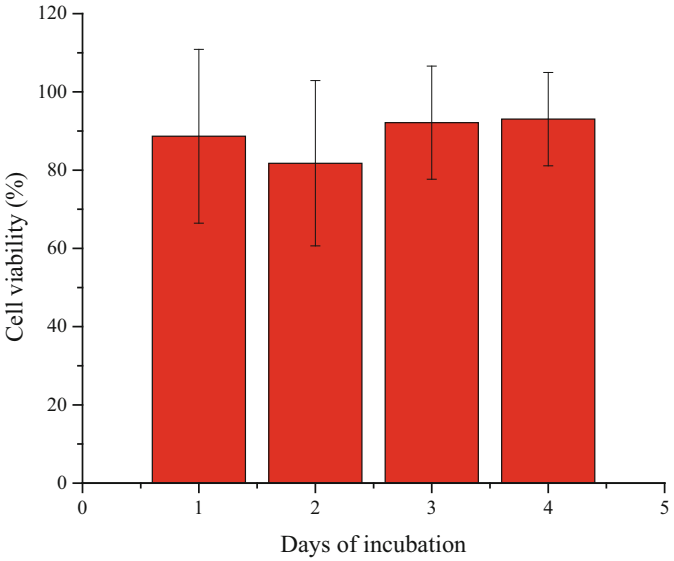

Fig. 3. Cell viability of the cells in tumor spheroids that have a growth in the average diameter, that is, those obtained in a culture medium without agarose and with a centrifugation time of 6 min. Viability is the average of 5 measurements of absorbance in the MTT assay with 100% viability control

Although there is an increase in the size, and therefore in the number of cells, the cell viability does not vary. The aggregates grow in diameter, but the proliferation zone no longer increases in size, because the oxygen and nutrients do not reach deeper areas of the aggregates. The necrotic zone causes that the cellular viability to decrease. It could even get to diminish more due to conditions like lack of oxygen in the center areas of the spheroid and depletion of the nutrients.

4 Conclusion

In conclusion, cancer cells were found to spontaneously form the spheroids in round-bottomed tubes when centrifugation time was varied from 6 min to 15 min. Agarose was used to evaluate the influence of these polymer on the compaction and growth of multicellular spheroids, and no significant differences were found in growth nor morphology of tumor spheroids. A bigger average size of the tumor spheroid was obtained in a medium lacking agarose that was centrifuged for 6 min. The growth of the spheroids is due to the aggregation of viable cells. However, the cell viability does not increase due to the formation of the necrotic zone. Therefore, the obtention of cancer spheroid described in this work would offer useful tool for nanomedicine applications.

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