Effects of Alginate-Gelatin Ratio and Lithium on SK- Hep1 Cell Proliferation Encapsulated in Bio-Polymeric Microspheres

Taqwa A. A. Qasem

Submitted to the
Institute of Graduate Studies and Research
in partial fulfillment of the requirements for the degree of

Master of Science
in
Chemistry

Eastern Mediterranean University
June 2020
Gazimağusa, North Cyprus

Approval of the Institute of Graduate Studies and Research

Prof. Dr. Ali Hakan Ulusoy
Director

I certify that this thesis satisfies all the requirements as a thesis for the degree of Master of Science in Chemistry.

Prof. Dr. İzzet Sakallı
Chair, Department of Chemistry

We certify that we have read this thesis and that in our opinion it is fully adequate in scope and quality as a thesis for the degree of Master of Science in Chemistry.

Asst. Prof. Dr. İmge Kunter
Co-Supervisor

Prof. Dr. Mustafa Gazi
Supervisor

Examining Committee

1. Prof. Dr. Mustafa Gazi
2. Prof. Dr. Elvan Yılmaz
3. Assoc. Prof. Dr. Mahmut Çerkez Ergören
4. Asst. Prof. Dr. Deniz Balcı
5. Asst. Prof. Dr. İmge Kunter

ABSTRACT

In comparison to 2D systems, 3D tumor culture systems are superior models to mimic the tumor behavior as *in vivo*. In order to understand the behavior of cells *in vitro* as they are *in vivo*, researchers are focusing on 3D systems and these systems are becoming popular as one of the hot techniques for cancer research. One of the aims of this study is to evaluate the effect of natural polymers alginate and gelatin and their ratios on liver adenocarcinoma cells SK-HEP1 spheroids. The second aim is to analyze the effect of lithium on SK-HEP1 spheroids size and number in various ratios of alginate and gelatin.

In this study, different concentrations of alginate and gelatin hydrogels were used with SK-HEP1 adenocarcinoma cell line to form multicellular tumor spheroids via the simple droplet-based method. Morphology and size of spheroids which indicates cell proliferation were evaluated using phase-contrast microscopy. Results revealed that gelatin increases cell proliferation without disturbing the morphology of beads up to 1.5% alginate and 0.5% gelatin ratios. Although gelatin supported cell proliferation and growth, over 0.5% of gelatin in biopolymer blend disturbed the bio-polymeric bead morphology. Lithium inhibited cell proliferation and decreased the size of cell spheroids. These results are in agreement with the effects of lithium on conventionally cultured SK-HEP1 cells in 2D systems. Our results indicated that cheap and abundant natural polymers such as alginate and gelatin could be potential candidates to create 3D culturing environments including microspheres for cancer.

Keywords: Alginate, Gelatin, Biopolymers, 3D Cell Culture, SK-HEP1 Spheroids.

ÖZ

In vivo tümör davranışını taklit etmekte, 2 boyutlu (2D) sistemlere göre birçok avantajı olan 3 buyutlu (3D) kültür sistemleri son yıllarda kanser araştırmacıları tarafından sıklıkla kullanılan yeni bir teknik olarak her geçen gün artarak kullanılmaya başlanmıştır. Çalışmamızın amaçlarından ilki; aljinat ve jelatin gibi doğal polimerlerin çeşitli oranlarda kullanılarak elde edilen biopolimerlerin karaciğer adenokarsinom hücresi SK-HEP1 sferoidleri üzerindeki etkisini değerlendirmektir. Çalışmanın diğer bir amacı ise, 2D sistemlerde etkisi bilinen lityumun, 3D lu ortamda oluşturulan SK-HEP1 sferoidleri boyutu ve sayısı üzerinde yaratacağı etkileri analiz etmektir.

Çalışmamızda, SK-HEP1 adenokarsinom hücre hattı ile basit damlacık bazlı yöntemle çok hücreli tümör küreleri oluşturmak için farklı aljinat ve jelatin hidrojel konsantrasyonları kullanılmıştır. Farklı koşullarda hücre proliferasyonu analizi, faz kontrast mikroskopisi ile sferoidlerin morfolojisi ve büyüklüğü değerlendirilerek yapıldı. Sonuçlarımız, jelatinin hücre proliferasyonunu arttırdığını gösterdi. Jelatin hücre proliferasyonunu ve büyümesini desteklese de, biyopolimer içindeki jelatinin % 0.5'inden fazlası biyo-polimerik boncuk morfolojisini bozdu. Lityum, 2D sistemlerde olduğu gibi, 3D'lu biyopolimerik jeller içerisinde de hücre proliferasyonunu inhibe etti ve hücre sferoidlerinin boyutunu azalttı. Sonuçlarımız, lityumun 2D sistemlerde geleneksel olarak kültürlenmiş SK-HEP1 hücreleri üzerindeki etkileri ile uyumludur. Bu tez kapsamında elde edilen sonuçlar, aljinat ve jelatin gibi ucuz ve bol miktarda bulunabilen doğal polimerlerin, kanser

araştırmalarında kulanılmak üzere, mikroküreler dahil 3D kültür ortamları oluşturmak için potansiyel adaylar olabileceğini göstermiştir.

Anahtar kelimeler: Aljinat, Jelatin, Biopolimerler, 3D Hücre Kültürleri, SK-HEP1 Sferoidleri.

ACKNOWLEDGMENT

I would like to show my appreciation and sincerest gratefulness to all the extraordinary individuals, who made composition this thesis conceivable and were engaged with the numerous features of this ongoing work.

It is with immense appreciation that I acknowledge the support and help of my supervisors Prof. Dr. Mustafa Gazi and Assist. Prof. Dr. İmge Kunter who were the reason behind this dissertation and my friends who supported me. I also would like to thank Prof. Dr. Elvan Yilmaz as she was my starting point in the polymer field.

Finally, the research that I have done at EMU would not have been possible without the support of my father Anwar, mother Intisar, grandmother Khitam, aunts Nahid and Hala and my uncles for their endless support, encouragement and love. Where would I be without you?

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

Chapter 1

INTRODUCTION

In the ongoing years, noteworthy advances in the field of nanotechnology, particularly in material science and biomedicine, have been accomplished [1, 2]. In particular, the clinical application of biomaterials in cancer study and treatment [3–5].

This study aimed at preparing hydrogel beads of alginate-gelatin to evaluate liver adenocarcinoma cells encapsulated in microspheres and analyzing the effect of lithium on these spheroids as a 3D environment for cancer cells culturing.

1.1 Biopolymers

Polysaccharides are natural polymers biocompatible [6–8] non-lethal, biodegradable [2] and non-toxic [6]. Biopolymers can be got from natural sources which they are economic, easy to get, rich and sustainable [8]. Biopolymers have got from creature tissues are especially famous since they hold comparative biochemical materials that cells involvement with their local tissue and may advance tissue recovery. Among the most normally utilized common polymers is gelatin. Biopolymers from non-mammal tissues for instance, alginate is also used for making 3D scaffolds [9].

Natural biomaterials have utilized cell conduct and capacity, empowering the progression of tissue building for remedial applications. To all the more likely copy the physical, biochemical, and physiological prompts of local tissues, regular

crossover 3D frameworks have widely been investigated [10]. The biocompatibility of these polymers is talked about as far as tissue reactions in both the host and matrix to suit the practical endurance of the cells [7]. Among biopolymers, alginate and gelatin are widely utilized for some biomedical applications because of their biocompatibility, and biodegradability [11]. The associations among carbohydrates and proteins have built by means of two essential synthetic responses prompting covalent holding, looking like the proteoglycans in the ECM [10].

1.1.1 Alginate Structure and Its Biomedical Applications

Alginate is a linear and water- soluble [8] biopolymer extracted from seaweed, for example, brown algae [12–14] and it is the most plentiful marine biopolymer and the most plentiful biopolymer on the planet after cellulose [15]. Alginate is a polyelectrolyte with two atomic building blocks (α- guluronic acid and β- mannuronic acid) , which manage its auxiliary characteristics and advance establishing ionic interaction with divalent cations to form ionically crosslinked gels [15, 16]. The building blocks of alginate are α- guluronic acid (G-block) and β- mannuronic acid (M-block) [14, 17, 18], (Figure 1).

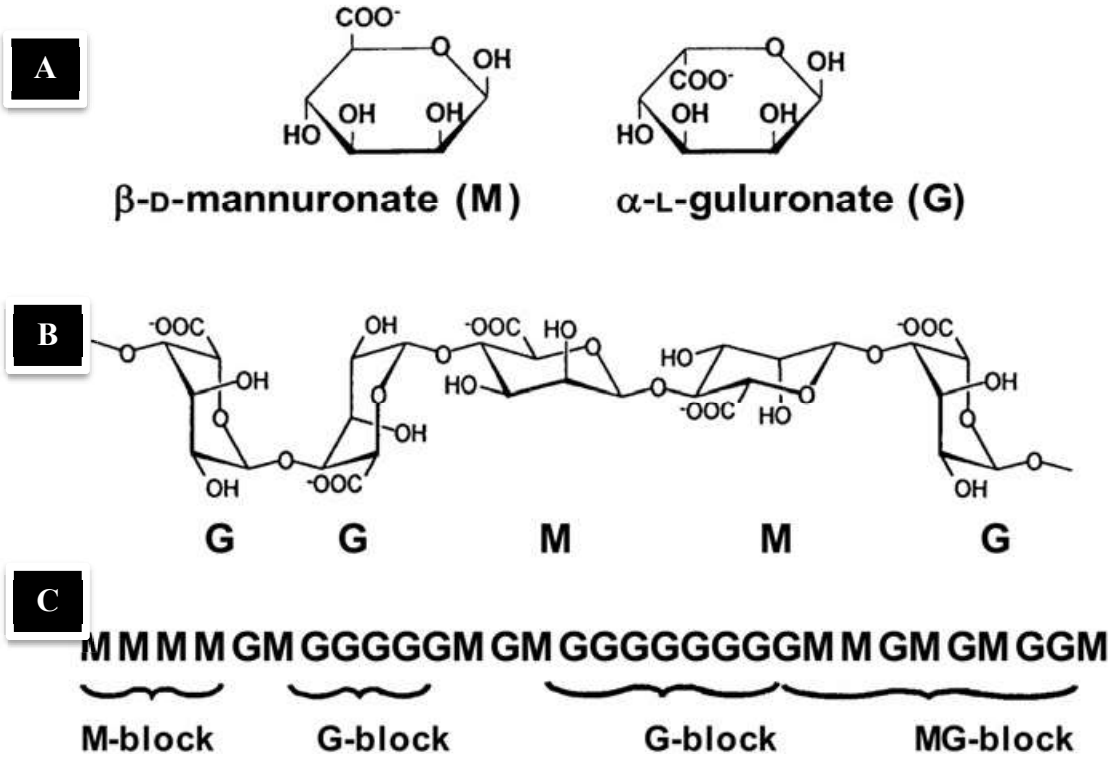

Figure1.1. Chemical structure of alginate: (A) alginate monomers, (B) chain adaptation, (C) block dissemination .

Alginate gels have for a long time been utilized as devices in both drug delivery system and cell encapsulation applications [19] in the bead structure for the most part delivered by dribbling alginate arrangement into a $CaCl_2$ bath [17, 20]. In the course of the most recent decades, alginates, normal beneficial polymers, have progressively attracted consideration as alluring compounds in the biomedical and pharmaceutical sectors because of their interesting physicochemical properties and adaptable organic functions [12, 17, 21].

Numerous regularly used materials got from extra-cell lattice made great frameworks for cell development [22]. Alginate is presently generally utilized as immobilizing materials for cells or tissue [23] in the improvement of counterfeit organs [15]. Alginate has become the most concentrated biomaterial for the exemplification of living cells [16]. Furthermore, it has been generally utilized as a natural biomaterial

in cell encapsulation for regenerative medication applications, as it is exceptionally biocompatible and has low immunogenic properties [24].

1.1.2 Gelatin Structure and Its Biomedical Applications

Gelatin is a promising material as a platform with helpful and regenerative attributes due to its synthetic similarities to the extracellular matrix (ECM) in the local tissues, biocompatibility, biodegradability, cost-viability, low antigenicity and plenitude [10]. Gelatin is a biodegradable protein, produced by acidic or fundamental hydrolysis of collagen, which includes breaking of the collagen's triple- helix structure into irregular curls [11, 25–32]. It consists of 19 amino acids [8] almost contains significant levels of hydroxyproline, proline and glycine, which can be broadly found in nature and is the significant constituent of skin, bones and connective tissue [1, 30]. Collagen might be obtained from different sources, including bovine, porcine, or fish through different strategies [10].

Gelatin is generally utilized in biomedical uses, for instance in tissue building, wound dressing, quality treatment, and drug delivery because of its high biocompatibility and biodegradability [33]. However, gelatin's poor mechanical properties, which limit its more extensive potential and medical applications, can be overcome by covalent crosslinking with different polysaccharides [26, 34, 35]. Gelatin is a characteristic and low- cost biopolymer whose properties, for example, negligible immunogenicity and apparent degradability, make it probably the best choice for tissue designing [2].

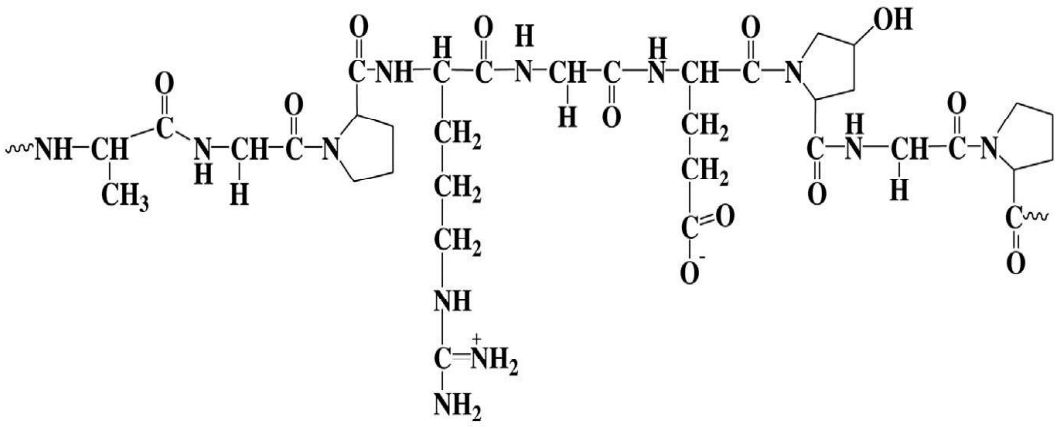

Figure1.2. Chemical structure of gelatin.

1.1.3 Hydrogels

There is developing gratefulness for the job that the extracellular matrix has an impact in controlling cell conduct. Mechanical, auxiliary, and compositional signs, either alone or in the show, can radically modify cell work. Biomaterials, and especially hydrogels, have been created and executed to display characterized subsets of these signals for exploring innumerable cell forms towards getting morphogenesis, maturing, and illness [19, 20, 37–40].

Hydrogels also coined as 'hungry networks' [41] are hydrophilic polymeric materials recognized by high water content [32] which may retain from 10–20% up to a huge number of times their dry load in water [30, 42] and assorted physical properties [27, 38, 43, 44]. Hydrogel arrangement includes the progress of fluid precursor arrangements into solid materials, which can be performed utilizing either physical (non-covalent) or chemical (covalent) crosslinking to gather the hydrogel parts [36, 37] and do not dissolve in the water [27, 32, 38]. Hydrogels are novel materials that display great biocompatibility and high penetrability for oxygen, supplements and other water-soluble metabolites to frame three-dimensional systems [16] to be a helpful strategy to create multicellular spheroid models [3, 19, 45].

Polymer hydrogels are viewed as appropriate for 3D cell culture as they have similitudes to characteristic extracellular matrix [46–48]. Moreover, they have demonstrated value in scope of cell culture applications, uncovering major wonders controlling cell conduct and giving devices to the development and coordinated separation of different cell types in manners impractical with regular culture substrates [31, 37]. Significant new disclosures in undifferentiated organism explore, cancer biology, and cellular morphogenesis have been acknowledged with model hydrogel frameworks commenced on these structures [5]. Most hydrogels comprise of micron/nanometer- estimated size that is regularly too little to even consider allowing post-creation cellularization and do not have the microtopography required for controlling the shape of cell and supporting cell portability, cell multiplication, and lattice creation. To overcome the above restrictions, many attempts have been made by utilizing hydrolytically and enzymatically degradable biopolymers. These hydrogels have segments that continuously break down and produce inner space to encourage framework creation and many other bioactivities for cell [9].

1.1.3.1 Alginate Hydrogels

Alginates have become a critical group of polysaccharides because of their utility in planning hydrogels at mild pH and temperature conditions, reasonable for delicate biomolecules like proteins and nucleic acids, and living cells [18, 21]. Properties of alginate hydrogels can be custom fitted through the modefication of free hydroxyl and carboxyl groups to control dissolution, hydrophobicity, and insert organic functional groups appropriate to cell adhesion and survival [10]. The chemical composition of alginate blocks assume a significant job in their ability to shape ionic gels [45]. The circulation and extents of monomers M and G along the alginate chain

determined mechanical, expanding and dispersion properties of the hydrogel [16, 49].

When multi-valent cations (for example $CaCl_2$) are joined to a water-based alginate arrangement, they tie contiguous alginate chains shaping ionic interchain spans that cause quick sol-gel progress perfect with the endurance of the captured cells [16]. The coupling zone between four G deposits of two distinct chains and a cationic regularly portrayed as an "egg-box model".(Figure 3), [17, 21, 49].

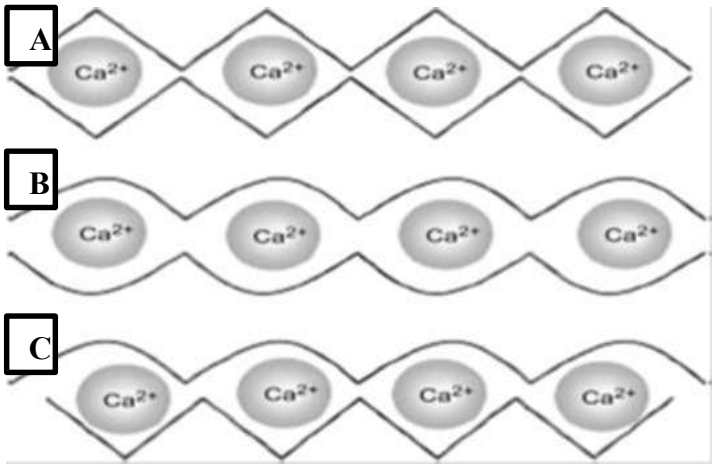

Figure1.3. Conceivable intersection focuses in alginate: (A) GG/GG junctions, (B) MG/MG junctions, (C) GG/MG junctions "egg-box model".

The significant explanation behind utilizing alginate gel as an implantation material is protection managed by the gel organized against the immune system of the host. The encapsulation innovation utilizing alginates is promising and gives a protected and straightforward strategy for embedding cells into different destinations of the human body [15, 48]. Cell encapsulation in alginate beads has been utilized for immobilized cell culture *in vitro* just as for immune-isolation *in vivo* [14, 50]. Ensnarement of living cells inside Ca-alginate circles is an entrenched method and can be completed in a solitary advance procedure under gentle situations. A cell

suspension is blended in with an (osmotically adjusted) sodium alginate arrangement, the blend trickled into a solution containing $CaCl_2$ and the beads momentarily structure gel-circles ensnaring the cells in a three-dimensional cross- section [17].

1.1.3.2 Gelatin Hydrogels

Gelatin–polysaccharide hydrogels may assimilate a lot of water, ordinarily over multiple times their dry mass, giving *in vitro* culture stages to investigate the conduct of mammalian cells in a lattice enlivened condition for tissue designing, preferring cell grip and development, penetration, and tissue vascularization [10]. Gelatin is a generally operated material to shape hydrogels for various biomedical applications, for instance, wound dressing, plasma expander, adsorbent material, and drug delivery as hard or delicate containers, hydrogels, or microspheres due to its high water content limit, biocompatibility, biodegradability furthermore, non-immunogenicity [31].

Gelatin has been an appealing contender for getting ready hydrogels utilized in long-term biomedical supplications [27] since it comprises of a huge number of functional groups and is effectively cross-linked [32]. Gelatin is effectively soluble in water at 37°C [26], non-immunogenic, and shows amphoteric behavior. Due to these advantages, gelatin-based hydrogels are used in the assembling of contact lenses, frameworks for tissue engineering, and drug delivery systems [28, 51, 52]. Gelatin-based hydrogels are fit for giving destinations to cell adhesion and proliferation [26]. Gelatin microspheres were utilized as a medication transporter for the parenteral drug delivery of malignant growth [50].

1.2 Cell Culture: A Short Review

The culture of tissues and cells is a basically and extensively used method that involves isolation of cells, tissues and organs from animals or humans and developing them in an *in vitro* or artificial condition [52– 54]. The expression culture implies to stay alive and develop in a suitable medium that types which would now be able to be developed in culture incorporate connective tissues, for example, fibroblasts, skeletal, cardiovascular also, smooth muscle, epithelial tissues, neural cells, endocrine cells and various kinds of tumor cells. The significant favorable position of using cell culture for any of these approaches is the consistency and reproducibility of results that can be gained from utilizing a bunch of clonal cells [52]. Cell culture plays a vital role in research because it gives fitting model frameworks to contemplating the standard physiology and natural chemistry of cells; [54, 55] the influences of medications and poisonous mixes on the cells, mutagenesis, and carcinogenesis [54, 56]. Moreover, it is exceptionally fundamental to biotechnology; the significant region of application of cell culture is in cancer research [52, 56]. Cell culture has become an irreplaceable apparatus to help reveal principal biophysical and bio molecular systems by which cells amass into organs and tissues, how these tissues capacity, and how that capacity becomes upset in disease [9].

Culture conditions differ broadly for every cell type, yet the artificial condition in which the cells are cultured perpetually comprises of an appropriate vessel containing a substrate or medium that provisions the basic supplements (amino acids, starches, nutrients, minerals), development elements, hormones, and gases (O_2, CO_2),

and controls the physicochemical condition (pH, osmotic weight, temperature) [52, 53, 57].

1.2.1 (2D) VS. (3D) Cell Culture

With the advancements in the field of cell science, biochemistry and medicine, it has become broadly acknowledged that conventional methods for controlling cells in a two-dimensional example have been inadequate for the need of present day medication. Today, expanding endeavors have been made to create three dimensional (3D) frameworks which better copy the geometry, science and flagging condition of regular extra cell matrix (ECM) [1].

Human body is three-dimensional drafts of action of cells with complicated cell-cell and cell-matrix associations and complex vehicle elements for supplements and cells [54]. Therefore, 3D cell culture is a significant device for biological research. When contrasted with the two-dimensional case, the three-dimensional (3D) cell culture system permits organic cells to proliferate or associate with their environmental factors in each of the three measurements [54, 56, 58]. Besides, 3D cell societies have more prominent security and more life expectancies than cell societies in 2D. Additionally, 3D aggregates can be refined for more, in any event up to a month, when contrasted with just about multi-week with 2D monolayer culture because of cell confluences [54]. Investigate has discovered that cells in the 3D culture condition differ physiologically and morphologically from cells in the 2D culture condition [58].

In most cases cell differentiation is acted in 2D in vitro. But this system does not give effective conditions to recreate the real usefulness of living tissues. Recently, various gatherings have advanced toward 3D culture to get progressively practical

biophysical and bimolecular situations following crucial instruments. The 3D association of cells, biomolecules, and biomaterials improves the arrangement of a particular microenvironment, permitting cell-to-cell correspondence, extracellular matrix (ECM) development, growth factor creation, and controlled dissemination of oxygen and supplements, just as waste item end, required after implantation [59].

For longer than a century, conventional (2D) cell cultures have been utilized as in vitro models to examine cell reactions to incitements from biophysical and biochemical signals. Despite the fact that these methodologies are all around acknowledged and have fundamentally progressed our comprehension of cell conduct, developing proof presently illustrates that, under certain conditions, the conventional frameworks can bring about cell bioactivities that go astray considerably the *in vivo* reaction. For example, some significant attributes of cancer cells can't be properly demonstrated in 2D societies. To overcome this limitation, 3D cell culture has constructed to mimic *in vivo* conditions [9], (Table 1.2).

Table1.2. Comparisons of 2D and 3D cell culture methods.

Type of culture	**2D**	**3D**	**Ref.**
Time of culture arrangement	Minutes to a few hours	A few hours to a few days	[3, 60, 61]
***In vivo* impersonation**	Do not mimic the original structure of tumor or tissue	Mimic *in vivo* structure	[62]
Culture quality	Straightforwardness of culture, reproducibility and high performance, long-term culture	cultures more hard to complete , awful performance and reproducibility, hard to decipher	[54, 63]
Characteristics of cells	Changed morphology and method for divisions; loss of assorted phenotype what's more, extremity	Save morphology and way of divisions, diverse phenotype and polarity	[64, 65]
Access to basic components	Unlimited access to supplements (unlike to *in vivo*)	Variable access to supplements (like *in vivo*)	[59, 66]
Cost of a culture	Cheap	More expensive	[67, 68]

1.2.2 (3D) Cell Culture in Cancer Research

Cancer is evaluated to have caused over 9.6 million deaths in 2018, despite everything being considered one of the significant reasons for death overall. Tumor types and tumor-invading cells are exceptionally heterogeneous, adding to the complexity of the disease [69]. Most malignancies emerge in people beyond 60 years old. As people are living longer and reaching older ages, malignancy is becoming a more frequently encountered medical issue [70].

Because of the rising demand for studying cells in the 3D system [55], the most widely recognized materials utilized are natural polymers because the control of their substance and properties, major for directing the grip and spreading of living cells and protein adsorption and thus cell connections are simpler to control [54]. 3D culture models consider as a multidisciplinary system that joining several key areas, specifically materials science, cell science, bioreactor structure, and adjusting these to clinical applications and research whenever expected for implantation [71].

Tumors have a three-dimensional (3D) structure, comprising of the cells, blood vessels, and extracellular substrates. Subsequently, *in vitro* malignant growth examines, it is fundamental to reenact however much as could reasonably be expected the 3D structure to get increasingly sensible discoveries. *In vitro* examinations are the initial step to look at the viability of anticancer medications and protection from them. Two-dimensional (2D) cell cultures are routinely utilized for this reason. In any case, 2D cell cultures suffer from the effects of various confinements, for example, the aggravation of associations between the cell and extracellular conditions and changes in cell morphology and extremity. In 3D situations, the cells develop are progressively like creature conditions [72]. The

spheroid group is especially helpful in malignant growth to inquire about as it empowers the fast revelation of morphological changes in changed cells [54, 55]. Spheroid cultures are widely applied to dissect the extracellular matrix (ECM) due to the closeness of spheroid societies and strong tumor behaviors [55]. Equal research likewise demonstrates that customary 2D cell culture techniques may not precisely imitate the 3D in vivo condition in which malignant growth cells reside as the 2D condition does not permit for territories of hypoxia, heterogeneous cell populaces, fluctuating cell expansion zones, ECM impacts, solvent sign slopes, and differential supplement and metabolic waste transport [73]. In addition to that, various investigations have discovered that cells refined in 3D models are more impervious to anticancer medications than 2D cultures [58]. Furthermore, one of the most evident contrasts in cells under 2D or on the other hand 3D cell culture frameworks is their adjusted multiplication limit. In 3D cell culture frameworks distinctive malignant growth cell lines showed adjusted – either diminished or expanded proliferation rates. Several cell lines indicated diminished proliferation rates in 3D frameworks [74]. In general, the morphological appearance of 3D spheroids is principally cell line subordinate [58].

It is important to grow better *in vitro* metastasis models of human malignant growth that will permit specialists to set up *in vitro* pre-testing techniques to mimic the *in vivo* microenvironment, which will spare costs, shorten test time, provide a much increasingly controllable environment, and reduce the loss of creature life [3, 45]. Past investigations show that the embodiment of liver malignant growth cells into ALG platforms may upgrade cell survival, proliferation, emission of ECM proteins and tumor malignancies [3]. Liver cancer is the 6th most continuous kind of disease and the fourth-leading reason for malignancy-related demise globally. The World

Health Organization has evaluated that more than 1 million individuals will pass on of liver malignancy in 2030, a projection that demonstrates a reasonable increment in rate and mortality [75].

Polysaccharides are the most ordinarily utilized regular polymers in cancer cell encapsulation. The purpose behind this is likely that polysaccharides consider the exemplification of cells under generally gentle conditions and generally do not meddle with the practical survival of the cells. Cells ought to develop and work in the polymer arrange as sufficiently as in their regular habitat [7]. Encapsulation of living cells, a type of cell immobilization, is one application for delivering counterfeit organs and cell treatment builds [45]. Cell encapsulation is utilized as a biotechnology device to tackle the mechanical issues got from handling and use of cells in an extraordinary scope of fields. This includes immobilization of the cells within a polymeric gel that allows the safeguarding of their metabolic action. Encapsulation into polymers is the most normally utilized cell immobilization innovation given its effortlessness and mellow working conditions [48].

The encapsulation of living mammalian cells inside a semi-porous hydrogel lattice is an alluring methodology for some biomedical and biotechnological applications, such as regenerative medicine and cell culture. Cell exemplification advancements target ensnaring practical and useful cells inside a semi-porous framework. A reasonable grid must be biocompatible, it must help cell endurance and thusly it must be porous to oxygen, to the approaching supplements and the active poisonous metabolites. Reasonable materials for cell encapsulation should imitate the extracellular network and ought to be handled under conditions perfect with the nearness of cells [16]. Encapsulation includes the envelopment of living cells in

polymer membranes to secure the cells from immune devastation. The presentation of this innovation goes back to 1933 when Bisceglie et al [76] considered the impact of embodiment on the endurance of tumor cells in the stomach cavity of pigs [7].

Spheroid is an *in vitro* malignancy cell imitation, which better than monolayer reflect regular tumor condition [77]. 3D spheroids are known to have zones of differential expansion. It has been indicated that cells at the fringe of tumor spheroid – that have better access to supplements and oxygen–divide more quickly [74].

Chapter 2

EXPERIMENTAL

2.1 Materials

2.1.1 Materials Used in Gel Formation

1. Sodium Alginate (average mol. Wt.: 80,000) was purchased from Alfa Aesar (Karlsruhe, Germany). Alginate was used to form gel beads which mimic the ECM as a suitable environment for cells.
2. Gelatin was purchased from (Aldrich, Germany). Another natural polymer was used to form gel beads which mimic the ECM as a suitable environment for cells.
3. Calcium Chloride was obtained from Sigma-Aldrich (Munchen, Germany). The calcium ions cross- linked the polymer.
4. Distilled water used to dissolve the polymers.
5. Magnetic Stirrer (ARE Velp Scientific) was utilized to mix the polymer solution.

2.1.2 Materials and Supplements Used in Cell Culture

Table 2.1. List of instruments and materials utilized in cell culture.

Instrument	Company	Purpose of use
Autoclave	Selecta AUTESTER ST DRY PV II 150	To maintain sterility
Esco's Cell Culture Co2 Incubator	ESCOGLOBAL	For incubating the cells in a body mimicking physical environment
Esco's Biological Safety Cabinets	Class II ESCO	For working in sterile conditions
Euromex Inverted Microscope	Euromex	For visualizing cells and evaluate beads morphology
CMEX 5.0 Camera	Euromex	For taking photos
Water Bath Bench-Top M 96 KP	Medical EXPO	To warm up the mediums and solutions
BOECO Centrifuge	C-28 BOECO Germany	For centrifuging cells and other solutions
Thermo Fisher Scientific Pipette Set	Thermo Fisher Scientific	To use exact volumes which they are lower than 1ml
Thermo Varioskan Flash	Thermo Fisher Scientific	For having photometric and flourometric measurements
Vortex Shaker	-	For mixing the solutions
Analytic Tare	BLC220 Boeco	For tarring the chemicals
Flat bottom 96 Sterile well plates	-	To keep the beads which contain cells in sterile conditions inside the incubator
Sterile disposable pipettes	-	Fundamental for each step of cell culturing
Glass Pasteur pipettes	-	For vacuuming the medium
Sterile 0.45uM injector filter	-	For filtering the polymers
Sterile Insulin Syringe	-	To make small beads

Table 2.2. Supplements used in cell culture medium.

Medium Supplements	Function
- DMEM (low glucose and high glucose)	A basal medium
- FBS (fetal bovine serum)	Growth supplement
- Penicillin- Streptomycin Mixture	To prevent bacterial contamination
- Trypsin (proteolytic enzyme)	To dissociate adherent cells from the plate in which they are being cultured
- Lithium Chloride	Chemical drug

2.2 Methods

2.2.1 Preparation of Cell Line

Human hepatocellular carcinoma cell lines of SK-HEP1 were provided by Izmir Biomedicine and Genome Centre. SK-HEP-1 were maintained in DMEM supplemented with 10% FBS, 100 U/mL penicillin, 2 mM L-glutamine, 100 mg/mL streptomycin and 1X NEAA at humidified 5% CO_2 at 37 °C temperature as an adherent cell and will be passaged when they reach enough confluency in 2D culturing systems before embedded in the hydrogel bead.

2.2.2 Preparation of Gel Beads

Before starting the experiment, it is mandatory to check if it was possible to make hydrogel beads using alginate- gelatin mixture. Firstly, gel beads were prepared using 0.75% (w/v) of alginate and 0.25% (w/v) gelatin by dissolving 0.075g alginate+ 0.025g gelatin in 10mL distilled water. Secondly, the same percentages of these polymers were carried out by dissolving them in 10mL of cell culture medium instead of distilled water to be sure if the medium and polymer gels were suitable for cell culture or not. Thirdly, 2% of $CaCl_2$ solution was prepared by dissolving 0.55g of $CaCl_2$ in 50mL distilled water as across linker to form gel beads. Fourthly, by using insulin syringe the polymer mixture was slowly dropped in the $CaCl_2$ bath for 1-2 min till the hydrogel beads formed. Finally, various concentrations of alginate-gelatin were prepared to start the experiment.

2.2.2.1 Preparation of Alginate- Gelatin Gel Beads with SK-HEP1

(Preliminary Experiment)

Alginate- gelatin hydrogels were prepared in different ratios by keeping alginate concentration constant at 0.75% (w/v) and changes were made on gelatin concentration. The process of preparation of these beads was carried out by dissolving 0.075g of alginate in 10mL distilled water to obtain a 0.75% (w/v) alginate solution. On the other hand, gelatin solutions were prepared in different concentrations by dissolving (0.025, 0.075, 0.125 and 0.15) g in 10mL distilled water to get (0.25, 0.75, 1.25 and 1.5) % (w/v) respectively. Then these viscous solutions were mixed to obtain six different ratios as concluded in (Table 2.3). Alginate dissolved at room temperature (RT), otherwise, gelatin did not. Therefore, polymer solutions were autoclaved at (121°C) for 15 minute by (Selecta AUTESTER ST DRY PV II 150 Autoclave) to dissolve the gelatin and sterilize the samples. Also,

2% of $CaCl_2$ was carried out by dissolving 0.55g/50mL distilled water .In addition to that, all other required materials as: (Glassware's, pipettes, flat bottom 96 well plates…etc.) were autoclaved to prevent any kind of possible contamination during cell culture process.

Table 2.3. Summary of gel ratios 1.

Sample No.	Alginate (%)	Gelatin (%)
1)	0.75	0
2)	0.75	0.25
3)	0.75	0.75
4)	0.75	1.25
5)	0.75	1.5

After sterilization, cell contains alginate- gelatin beads were prepared under sterile conditions inside Class II Biological Safety Cabinet (ESCO) as represented in (Figure 2.1) by following steps:

1. SK- HEP1 cells were counted under the microscope using Hemacytometer (Neubauer Champer).
2. Each sample of gel were mixed with a (5*10^4 cells/mL).
3. Magnetic stirrer (ARE Velp Scientific) was utilized for making proper beads shape.
4. Sterile insulin syringe was used and the polymer- cells solution was dropped slowly in the $CaCl_2$ bath on the stirrer. In the wake of being drenched in the $CaCl_2$ bath for 1-2 min, the prepared beads formed gels.

5. The $CaCl_2$ solution was discarded and the beads were washed twice with 5mL of phosphate buffer saline (PBS).
6. The cells were moved to a flat bottom 96 sterile well plate which includes cell culture medium (0.2mL of medium in each well). The medium was changed at regular intervals.

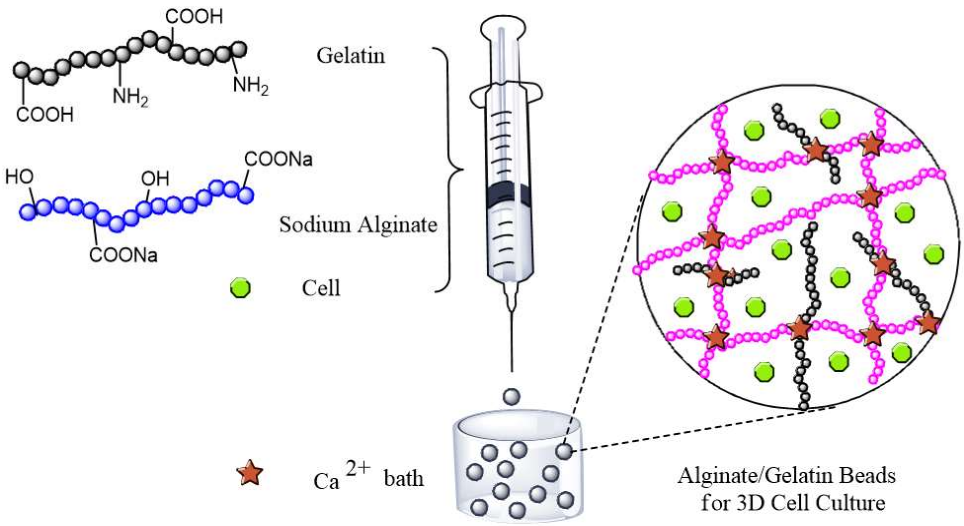

Figure2.1. Schematic illustrations for production of Alginate/Gelatin Beads and 3D cell encapsulation.

(Second Experiment)

Instead of preparing each polymer in 10mL distilled water separately then mixing them together, the decision was to add both of them in the same amount of water to get new ratios of these polymers as summed up in (Table 2.4).

Table 2.4. Summary of gel ratios 2.

Sample No.	Alginate (%)	Gelatin (%)
1)	1.5	0
2)	1.5	0.5
3)	1.5	1.5
4)	1.5	2.5
5)	1.5	3.5

The first experiment showed us that adding gelatin in high concentrations disturbed the beads shape. To overcome this issue, the percentage was changed. The decision was to decline the percentage of gelatin carried out while alginate concentration was the same. This experiment was indicated some problems over 1.5% gelatin therefore, a third experiment was designed.

(Third Experiment)

To overcome the problem of beads morphology in the previous trials, we decided to decrease gelatin concentrations 10x (ten times) by keeping alginate concentration the same. Gel preparation concentrations for the third trial are summarized in (Table 2.5). Moreover, two types of the medium had used; high glucose medium and low glucose medium to know the effect of a nutrient on cell proliferation.

Table 2.5. Summary of gel ratios 3.

Sample No.	Alginate (%)	Gelatin (%)
1)	1.5	0
2)	1.5	0.05
3)	1.5	0.15
4)	1.5	0.25
5)	1.5	0.35
6)	1.5	0.45

The alginate-gelatin hydrogel beads were prepared by the above given method using various concentrations of biopolymers. Alginate concentration was left the same as in the previous experiment at 1.5% (w/v). Otherwise, gelatin concentrations declined ten times to be; 0.05%, 0.15%, 0.25%, 0.35% and 0.45%.

2.2.3 Instrumental Analysis

2.2.3.1 Inverted Microscope Analysis

Photos of samples were obtained by Euromex inverted microscope (Euromex Company) by using CMEX 5.0 camera for visualizing and capturing the photos of the beads morphology and proliferation of cells.

Chapter 3

RESULTS AND DISCUSSION

3.1 Formation of Alginate-Gelatin Gel Beads

Alginate- gelatin beads were successfully prepared in various concentrations by a simple droplet-based method. It was observed that strong, uniform and spherical beads were formed as shown in (Figure 3.1).

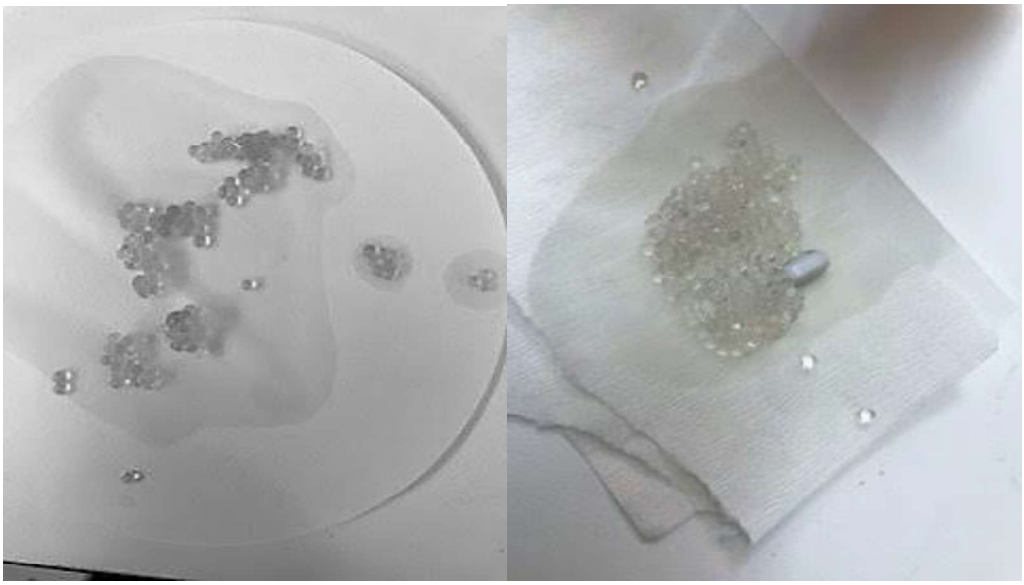

Figure 3.1. Optical photographs of alginate- gelatin beads.

3.1.1 Formation of Alginate- Gelatin Gel Beads with SK-HEP1

(Preliminary Experiment Results)

Alginate- gelatin gel beads were prepared under sterile conditions. Beads morphology, cell viability and proliferation of the SK- HEP1 were determined by studying the impact of various concentrations of alginate- gelatin hydrogel beads. It was noticeable that improper beads shape was formed at high gelatin concentration, because alginate interacted with gelatin more than with $CaCl_2$. Otherwise, the best cell proliferation was observed at a higher percentage of gelatin. The ratios of this experiment illustrated in (Figure 3.2).

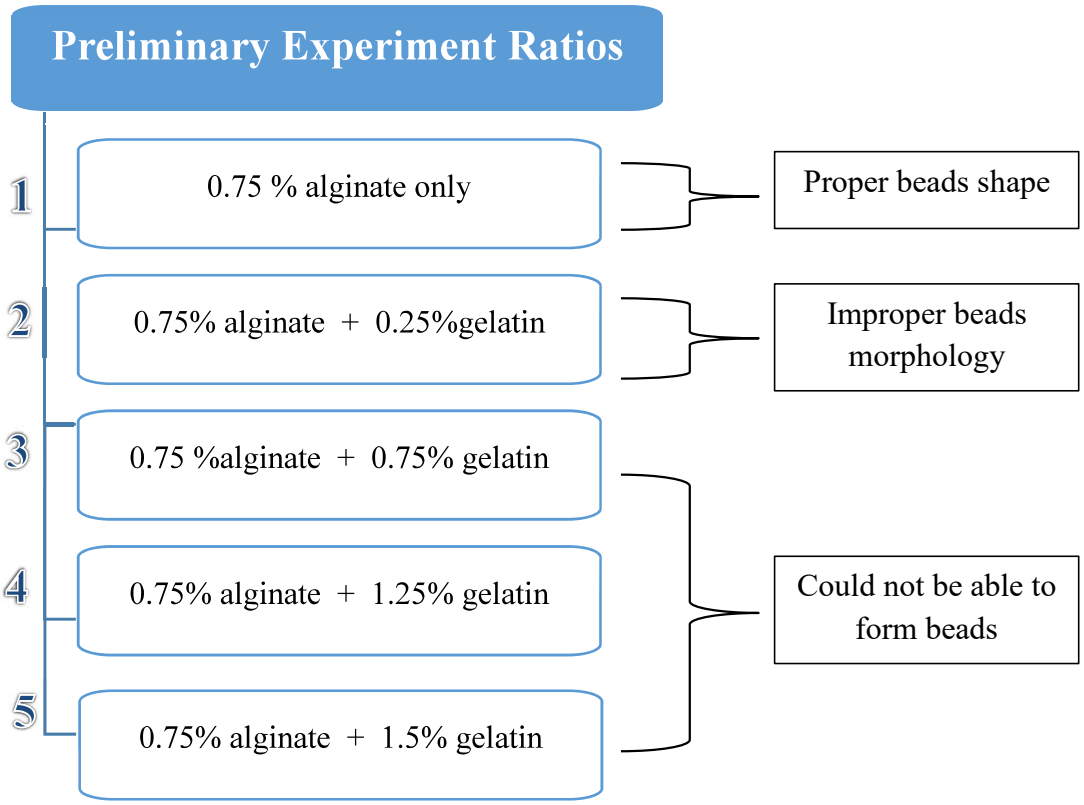

Figure 3.2. Ratios of polymeric hydrogels of the preliminary experiment.

Sample 1 (0.75% alginate- 0% gelatin) formed a well shape beads but as we increased the concentration of gelatin, beads were become amorphous. However, cell proliferation was encouraged with higher gelatin in sample 2 (0.75% alginate+ 0.25% gelatin) on the 7th day of cell culture as illustrated in (Figure 3.3). On the other hand, it was difficult to form beads at 0.75%, 1.25% and 1.5% gelatin with 0.75% alginate.

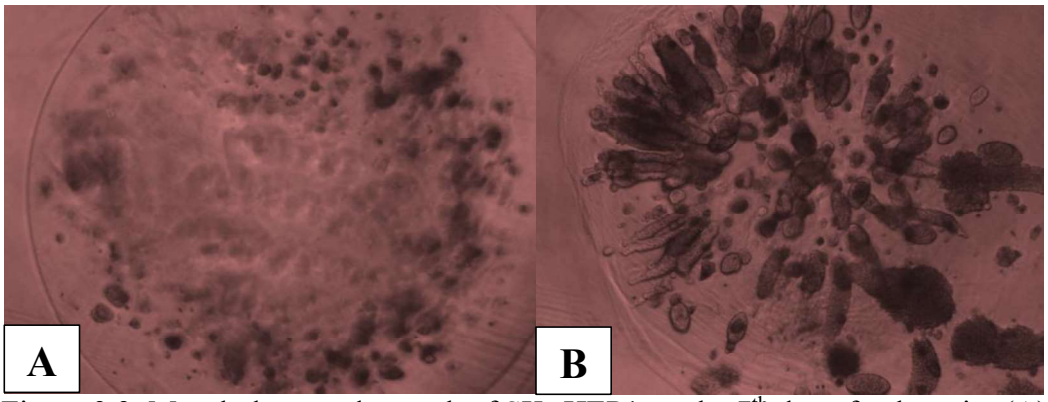

Figure 3.3. Morphology and growth of SK- HEP1 on the 7th day of culture in: (A) 0.75% alginate only. (B) 0.75% alginate + 0.25% gelatin of the Preliminary Experiment.

(Second Experiment Results)

In the previous preliminary experiment even gelatin had enhancing effect on cell proliferation, but it formed amorphous shape of the beads. Therefore, it was repeated in a different manner by using the same ratios with various percentages. Instead of dissolving each polymer in 10ml distilled water separately and then mixing them together, the gels prepared by adding 1.5% (w/v) of alginate with various concentrations of gelatin (0.5%, 1.5%, 2.5% and 3.5%) in 10 mL distilled water together not in separate as shown in (Figure 3.4). Results revealed that even the percentage of both polymers increased from 0.5 % to 1.5 %, over 1.5 % of gelatin disturbed the structure of the beads. These results support the preliminary experimental data that spheroids of SK-HEP 1 cells were ascended with increasing gelatin concentration. Even beads morphology was shapeless, but the proliferation of SK-HEP 1 cells was better at high gelatin concentration.

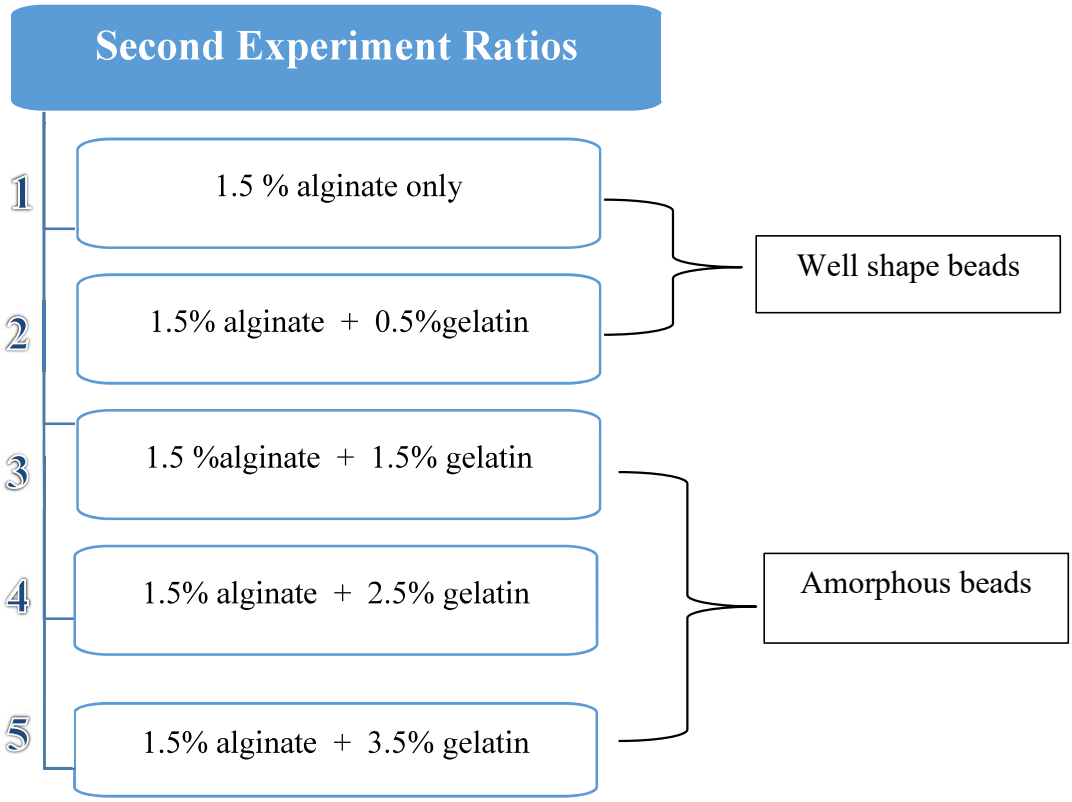

Figure 3.4. Ratios of polymeric hydrogels of the second experiment.

In this experiment, gelatin did not have the same impact in the second sample as in the previous trial. Nevertheless, in the third and fourth samples; (1.5%alginate+ 1.5% gelatin and similar alginate amount+ 2.5% gelatin) individually, gelatin defiantly increased cell proliferation.

During culturing the cells in 3D beads representative photos have taken by inverted microscope (Euromex) on the 7th day of culture to evaluate the morphological features and cell proliferation. It was noticeable, that gelatin had an effective impact on SK- HEP1 proliferation, especially at higher concentrations and specifically when the ratio was 1:1 (sample no. 3) as represented in (Figure 3.5). Maybe because the cancer cells divided in a higher rate than normal cells and it needs more energy, therefore gelatin considers a source of energy as it consists of many essential amino acids.

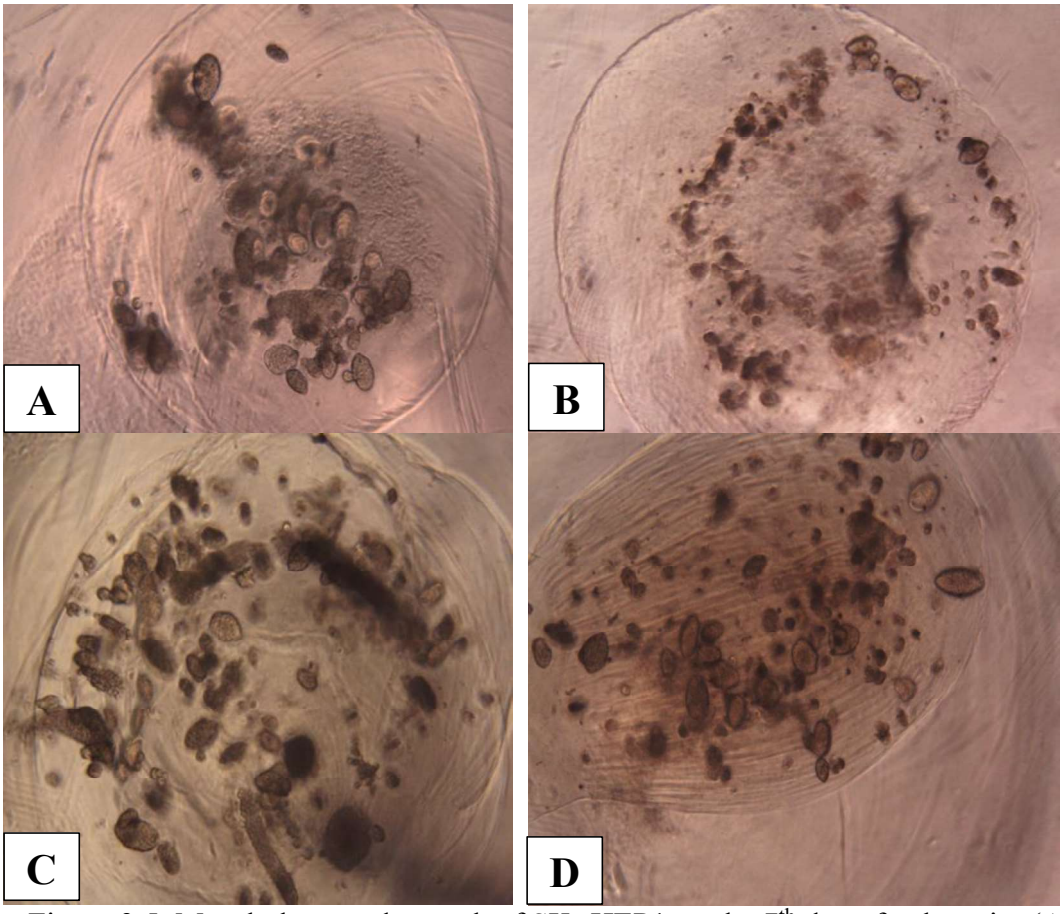

Figure 3.5. Morphology and growth of SK- HEP1 on the 7th day of culture in: (A) 1.5% alginate only, (B) same level alginate + 0.5% gelatin, (C) normal level alginate + 1.5% gelatin, (D) same concentration alginate + 2.5% gelatin.

(Third Experiment Results)

To be able to avoid destructive effect of the gelatin at bead morphology gelatin ratios were decreased ten times while alginate concentration kept same as shown in (Figure 3.6). Also the effects of high and low glucose medium on the proliferation of cell spheroids were investigated. Moreover, lithium (chemical drug) was added on day 8 of the culture to evaluate the inhibitory effect on the SK- HEP1 cells proliferation in 3D as on conventionally cultured (2D systems). The results visualized by taking photos between days 3-19 of culture and the impact of lithium evaluated by spheroid size and shape from day 8 till day 19 of culture.

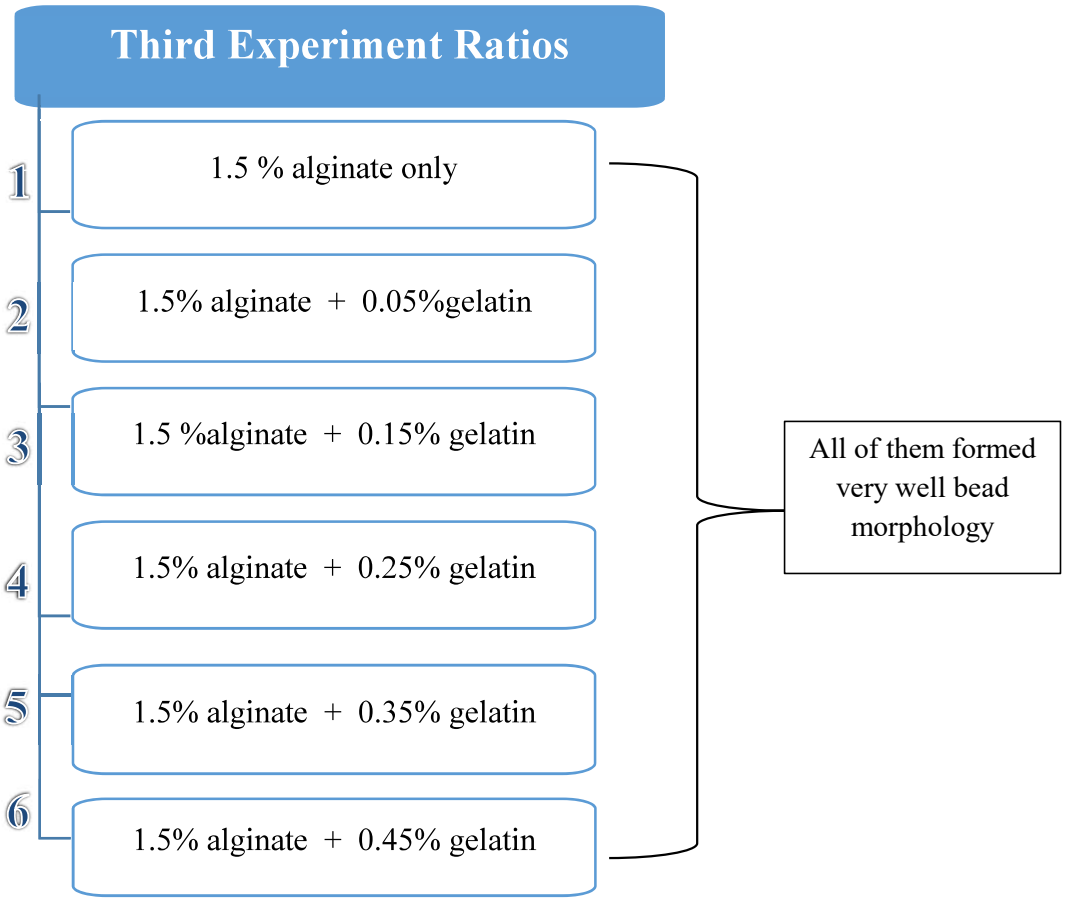

Figure 3.6. Ratios of polymeric hydrogels of the third experiment.

On day 3 of culture representative photos have taken for both type of medium; High glucose medium and low glucose medium as illustrated in (Figures 3.7 and 3.12 respectively). Although the 3rd day is too early to assess the spheroid size, it was obvious that increasing gelatin concentrations made the spheroids slightly bigger in both medium. At low glucose concentration the size of the spheroids in all conditions was slightly bigger than in high glucose medium.

At the day 8 of culture, the results were nearly same as day 3. Only at sample no.5 (1.5% alginate+ 0.35% gelatin) in high glucose spheroids sizes were bigger but numbers of spheroids were lower as represented in (Figure 3.8). The possible explanation for this situation is that growing spheroids could fuse to each other.

On day 11, the polymeric spheroids became bigger, particularly at higher gelatin concentrations. It was noticeable that gelatin had a great effect on SK-HEP1 cell proliferation as represented in (Figures 3.9 and 3.14).

The difference in spheroids morphology and proliferation between low glucose and high glucose mediums was obvious on day 15 and 19 of culture at high gelatin concentrations. At the first days of culture, low glucose condition had better effects on spheroids size and cell proliferation. On the other hand, after 15-19 days of the culture in high glucose medium, the spheroids size was interestingly bigger, especially in samples 5 and 6 where the gelatin concentration was the highest (0.35% and 0.45% individually) as shown in (Figures 3.10+ 3.11 at high glucose and 3.15+ 3.16 at low glucose).

3.1.2 Representative Photos of Third Experiment

3.1.2.1 Spheroids in High Glucose Medium

Day3 of Culture

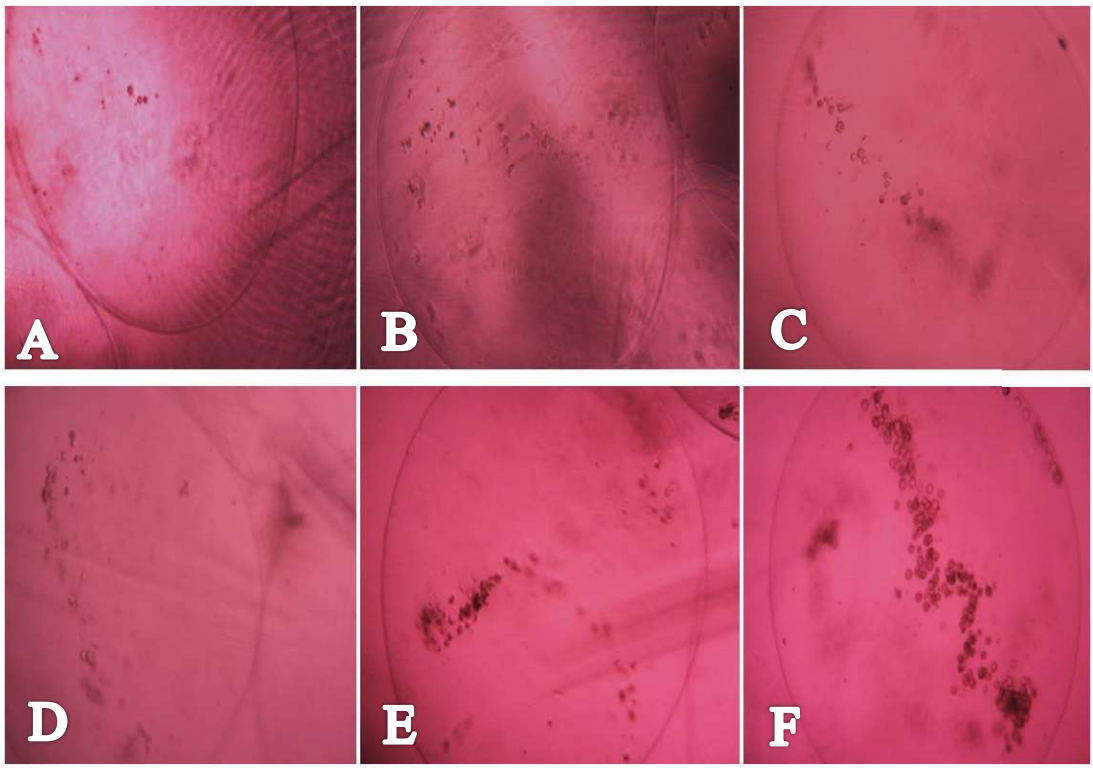

Figure 3.7. Morphology and growth of SK- HEP1 in high glucose medium on day 3 of the culture in: (A) 1.5% alginate only, (B) similar alginate ratio +0.05% gelatin, (C) same concentration alginate +0.15% gelatin, (D) exact amount alginate +0.25% gelatin, (E) normal concentration alginate +0.35% gelatin, (F) same level of alginate +0.45% gelatin.

Day 8 of Culture

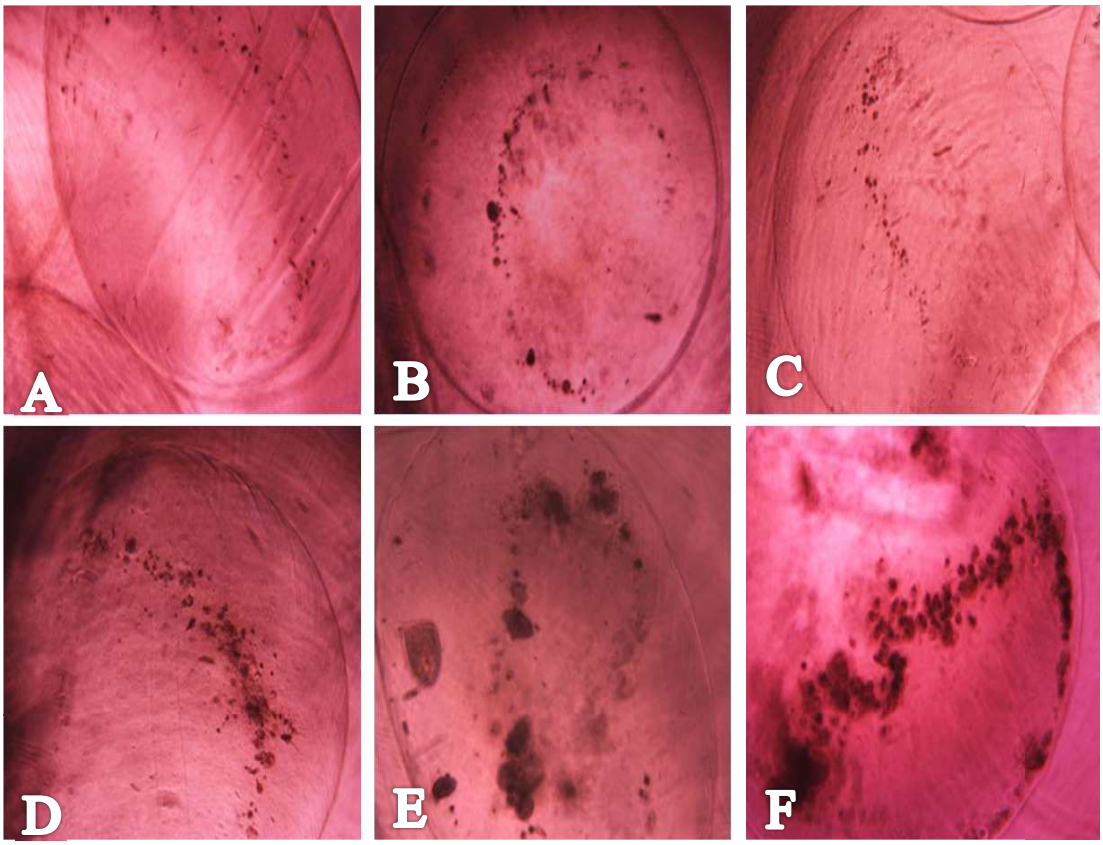

Figure 3.8. Morphology and growth of SK- HEP1 in high glucose medium on day 8 of the culture in: (A) same previous ratio alginate only, (B) normal amount alginate +0.05% gelatin, (C) similar concentration alginate +0.15% gelatin, (D) same level alginate +0.25% gelatin, (E) constant concentration alginate +0.35% gelatin, (F) similar amount alginate +0.45% gelatin.

Day 11 of Culture

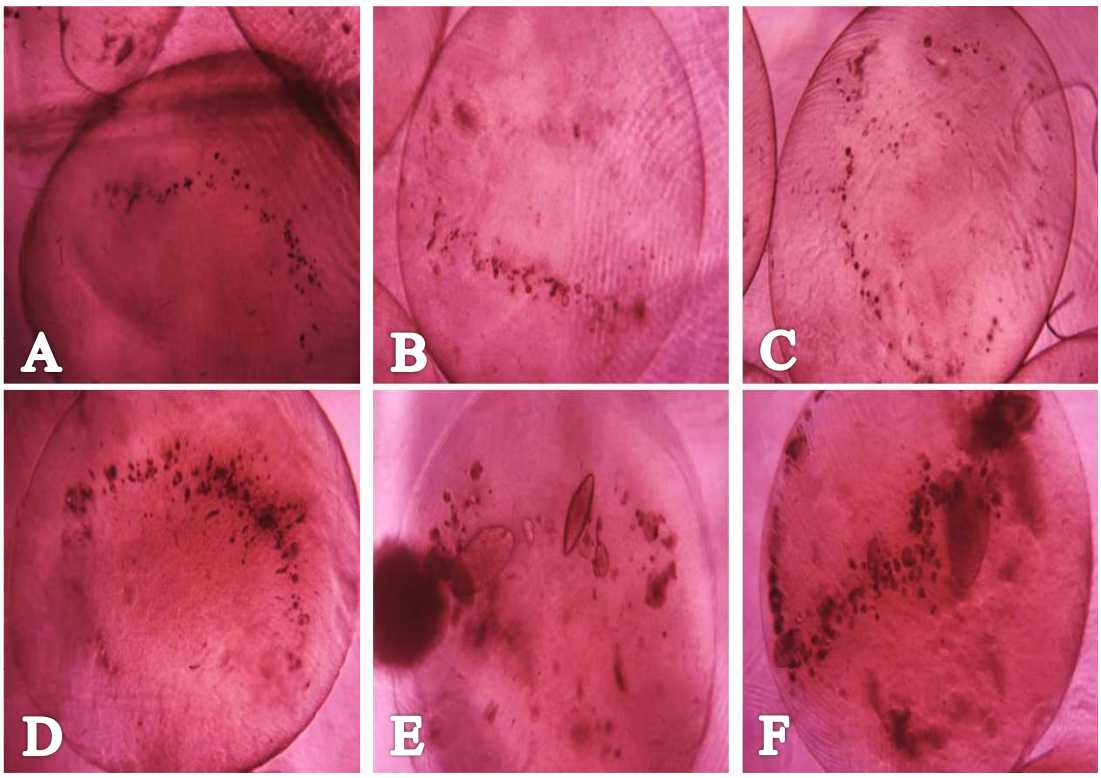

Figure 3.9**.** Morphology and growth of SK- HEP1 in high glucose medium on day 11 of the culture in: (A) exact previous concentration alginate only, (B) exact level alginate +0.05% gelatin, (C) constant percentage alginate +0.15% gelatin, (D) normal percentage alginate +0.25% gelatin, (E) same amount alginate +0.35% gelatin, (F) exact ratio alginate +0.45% gelatin.

Day 15 of Culture

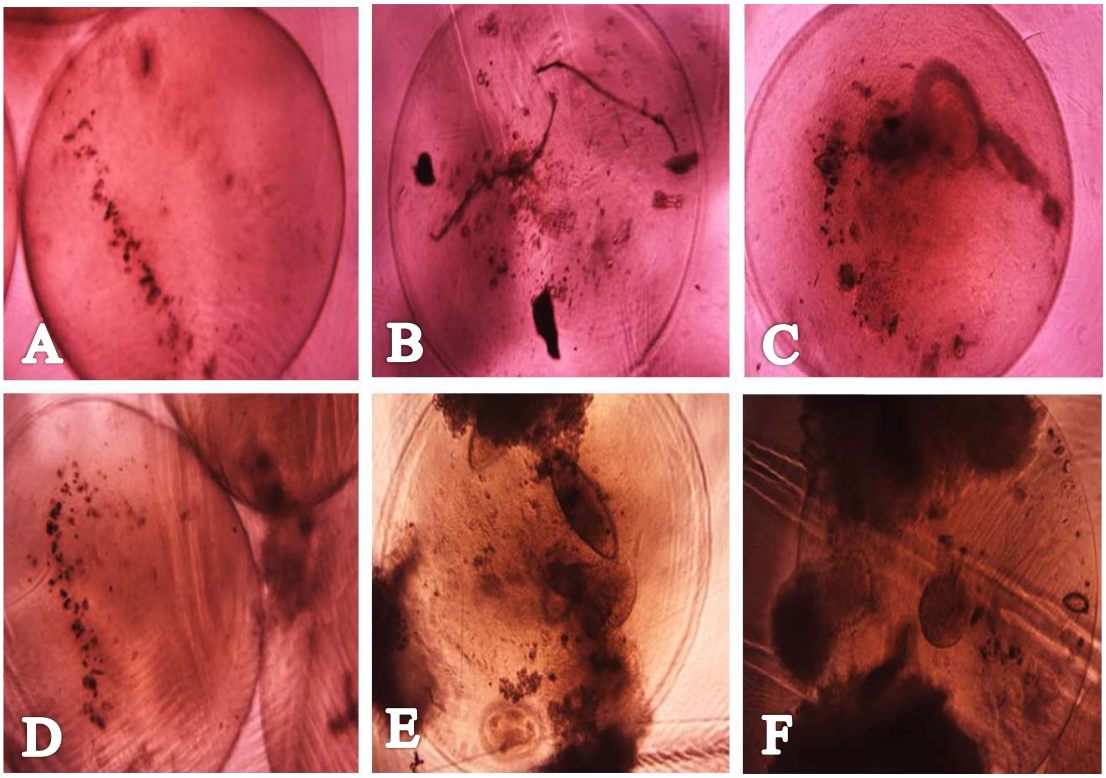

Figure 3.10. Morphology and growth of SK- HEP1 in high glucose medium on day 15 of the culture in: (A) Normal alginate concentration only, (B) same ratio alginate +0.05% gelatin, (C) similar amount alginate +0.15% gelatin, (D) exact level alginate +0.25% gelatin, (E) normal percentage alginate +0.35% gelatin, (F) same alginate concentration +0.45% gelatin.

Day 19 of Culture

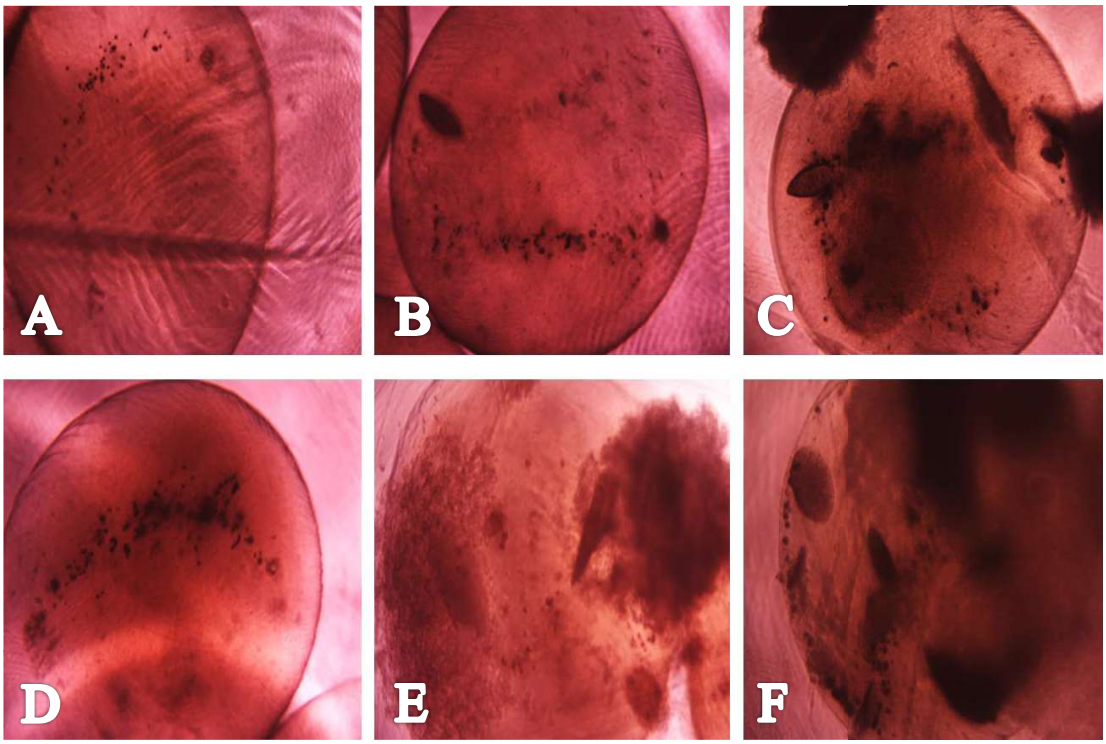

Figure 3.11. Morphology and growth of SK- HEP1 in high glucose medium on day 19 of the culture in: (A) 1.5 percent of alginate only, (B) similar percentage of alginate +0.05% gelatin, (C) exact level alginate +0.15% gelatin, (D) constant ratio alginate +0.25% gelatin, (E) same concentration alginate +0.35% gelatin, (F) similar alginate level +0.45% gelatin.

3.1.2.2 Spheroids in Low Glucose Medium

Day 3 of Culture

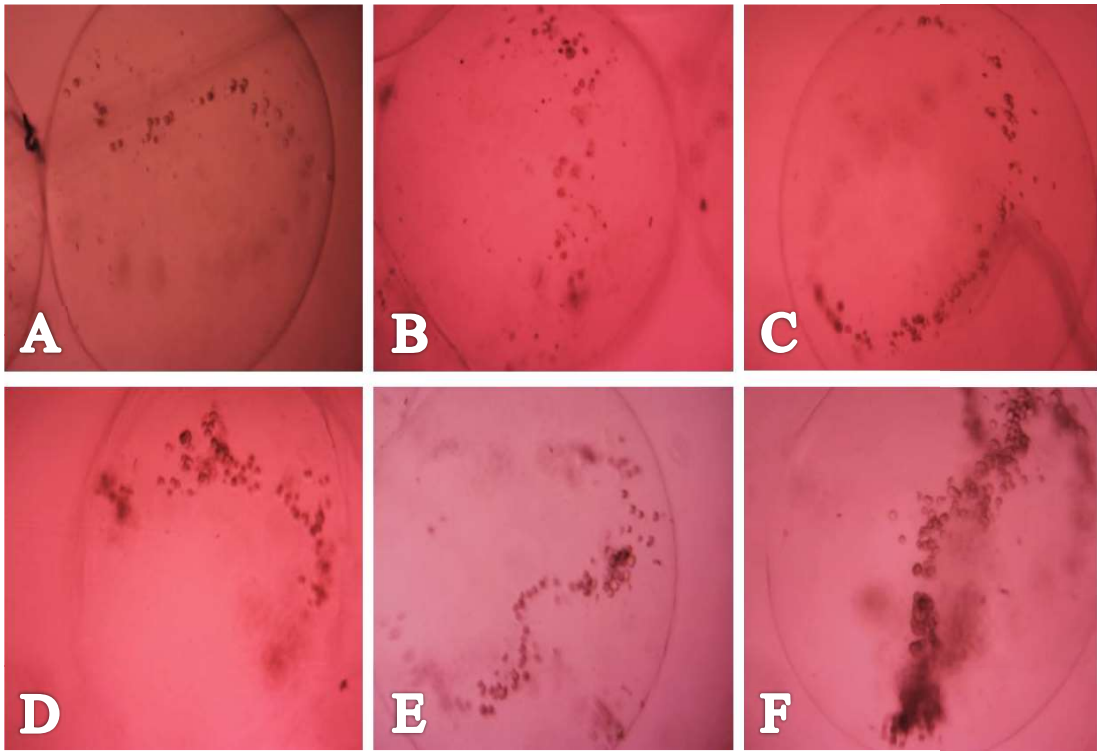

Figure 3.12. Morphology and growth of SK- HEP1 in low glucose medium on day 3 of the culture in: (A) normal alginate percentage only, (B) same normal alginate ratio +0.05% gelatin, (C) similar alginate concentration +0.15% gelatin, (D) exact alginate ratio +0.25% gelatin, (E) same alginate amount +0.35% gelatin, (F) exact alginate level +0.45% gelatin.

Day 8 of Culture

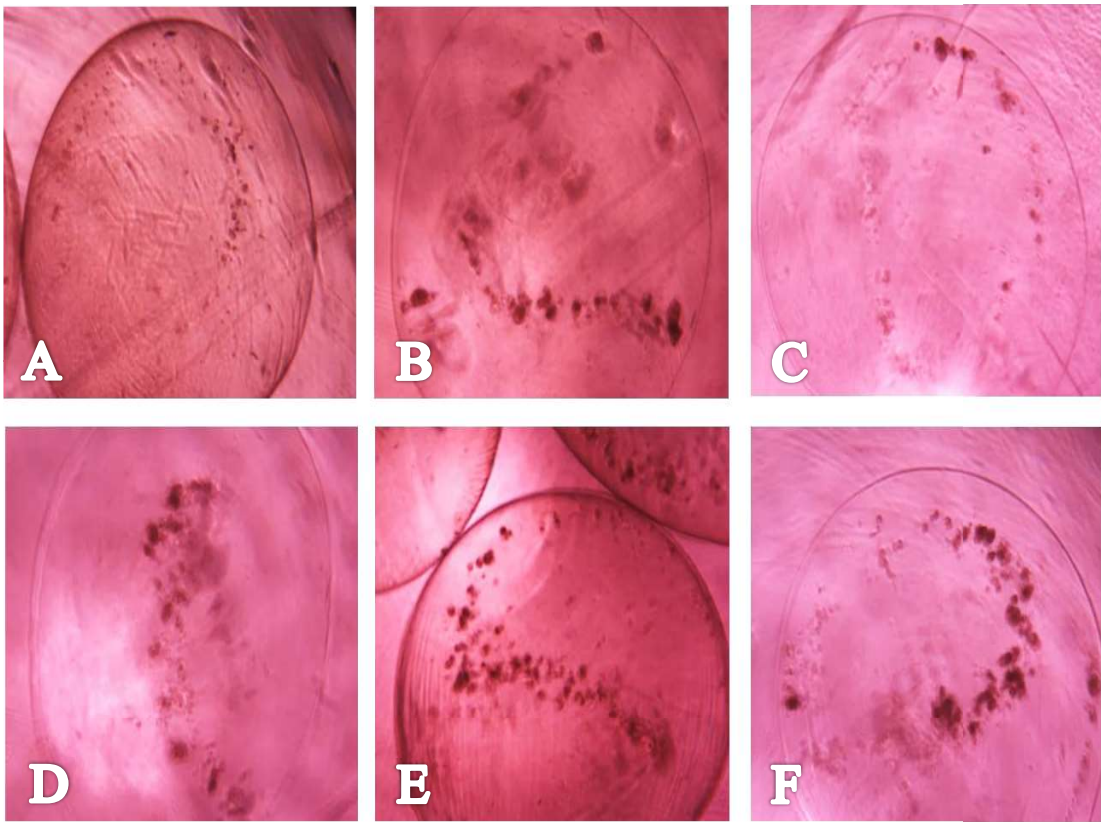

Figure 3.13. Morphology and growth of SK- HEP1 in low glucose medium on day 8 of the culture in: (A) similar alginate percentage only, (B) normal alginate level +0.05% gelatin, (C) same alginate ratio +0.15% gelatin, (D) similar alginate level +0.25% gelatin, (E) constant alginate concentration +0.35% gelatin, (F) normal alginate ratio +0.45% gelatin.

Day 11 of Culture

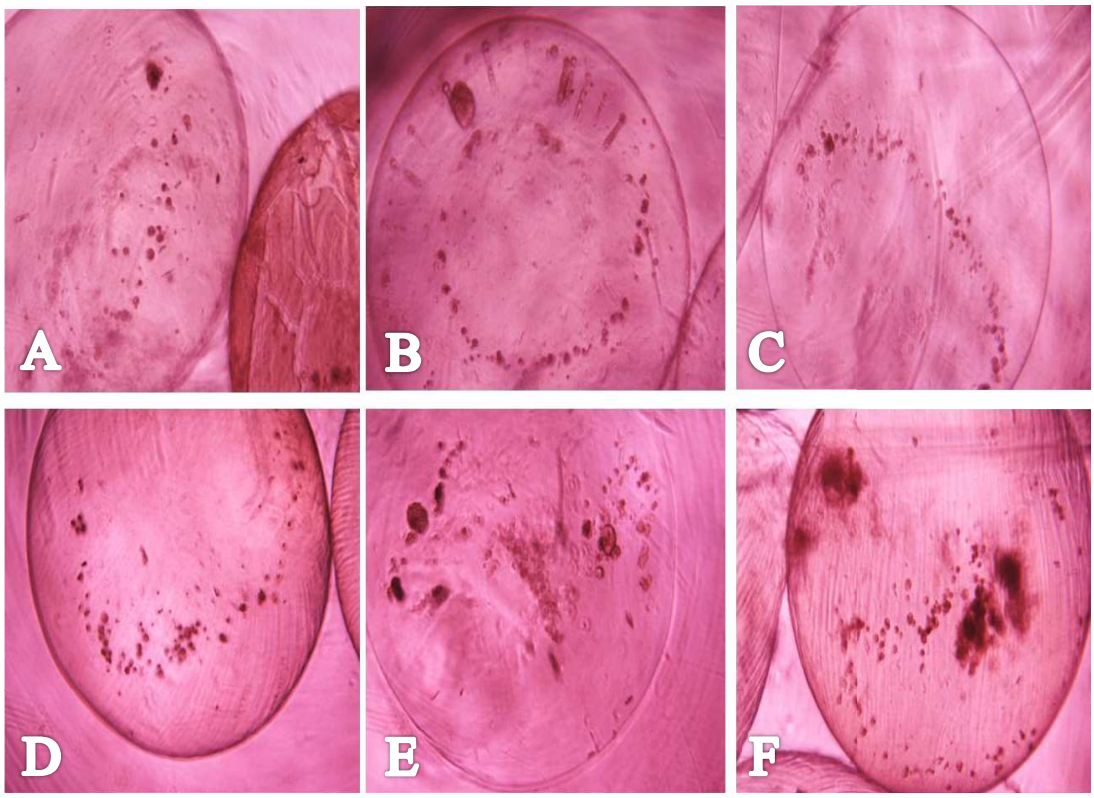

Figure 3.14. Morphology and growth of SK- HEP1 in low glucose medium on day 11 of the culture in: (A) similar previous level alginate only, (B) normal concentration alginate +0.05% gelatin, (C) exact ratio alginate +0.15% gelatin, (D) same alginate amount +0.25% gelatin, (E) similar percentage alginate +0.35% gelatin, (F) same ratio alginate +0.45% gelatin.

Day 15 of Culture

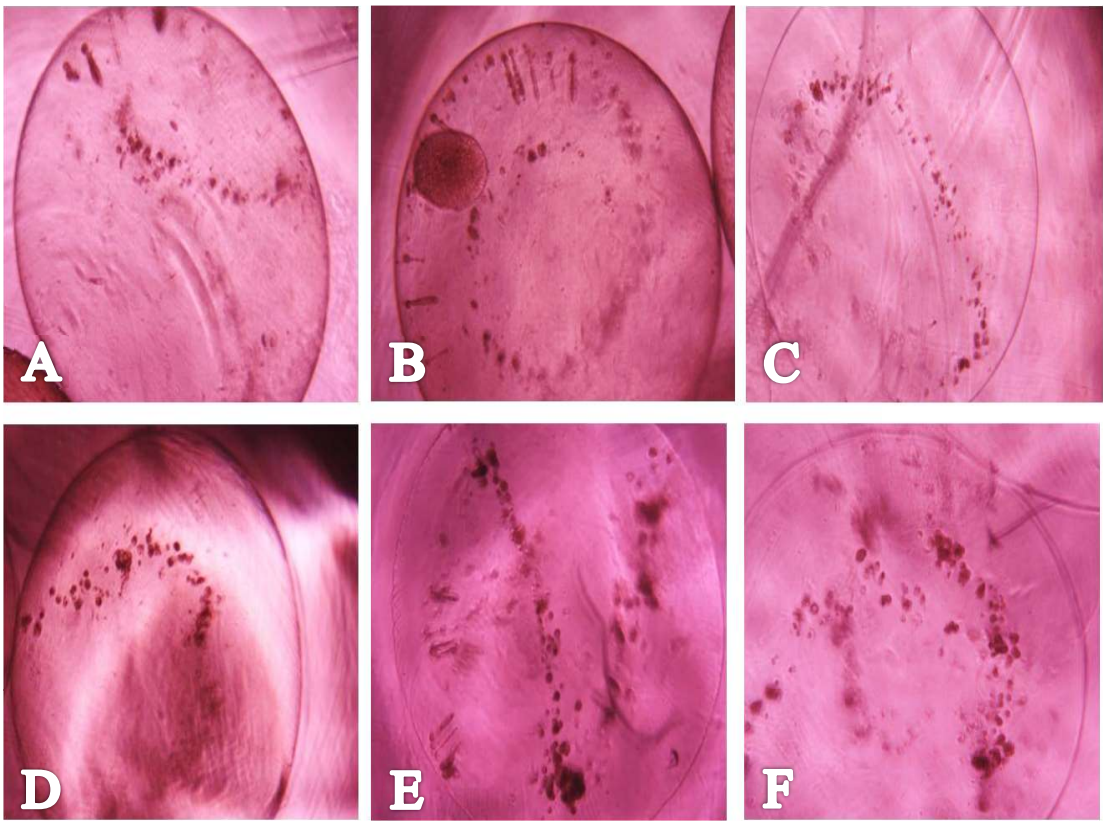

Figure 3.15. Morphology and growth of SK- HEP1 in low glucose medium on day 15 of the culture in: (A) normal amount alginate only, (B) exact alginate ratio +0.05% gelatin, (C) same alginate percentage +0.15% gelatin, (D) similar concentration alginate +0.25% gelatin, (E) normal percentage alginate +0.35% gelatin, (F) same normal ratio alginate +0.45% gelatin.

Day 19 of Culture

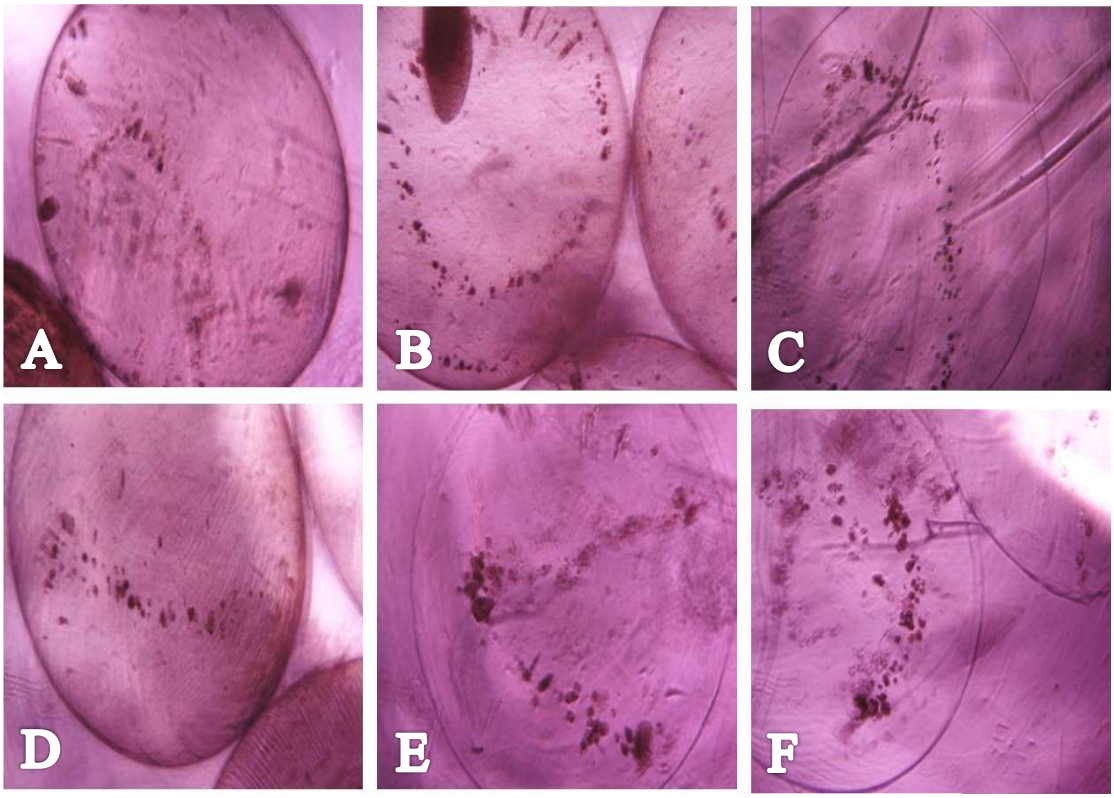

Figure 3.16. Morphology and growth of SK- HEP1 in low glucose medium on day 19 of the culture in: (A) exact level alginate only, (B) normal alginate concentration +0.05% gelatin, (C) similar alginate level +0.15% gelatin, (D) exact alginate percentage +0.25% gelatin, (E) same alginate ratio +0.35% gelatin, (F) normal alginate amount +0.45% gelatin.

3.1.3 The Effect of Lithium on Cell Spheroids in Different Conditions

LiCl was added to the cell culture medium after creating the cell spheroids (the amount of chloride is not that much effective). The cell spheroids were created with various concentrations of alginate and gelatin as in the previous experiments. After that, they cultured for 8 days to be able to reach small cell blocks then, lithium was added to the cell culture medium. It is known that LiCl had a suppressing effect on SK-HEP1 cell proliferation in 2D culture [78]. In our experiment, the impact of LiCl was clear as well in 3D cell culture.

Lithium inhibited cell proliferation and decreased the size of cell spheroids in both medium and in all conditions as represented in (Figures 3.17, 3.18 and 3.19 at high glucose & 3.20, 3.21, 3.22 at low glucose). Even the lithium had an inhibition effect on cell proliferation according to the spheroid size in all conditions, but its impact was lower at high gelatin concentrations (0.35% and 0.45%) may be because they were already at a big size before adding the lithium.

It is not astonishing that tumor cells are less sensitive to certain chemicals in 3D than in 2D culture. This impact might be brought about by diminished access to blends in the medium or by pathophysiological contrasts because of hypoxia, or by changes in the cycle of cell [62].

3.1.3.1 Spheroids and Lithium Effects in High Glucose Medium

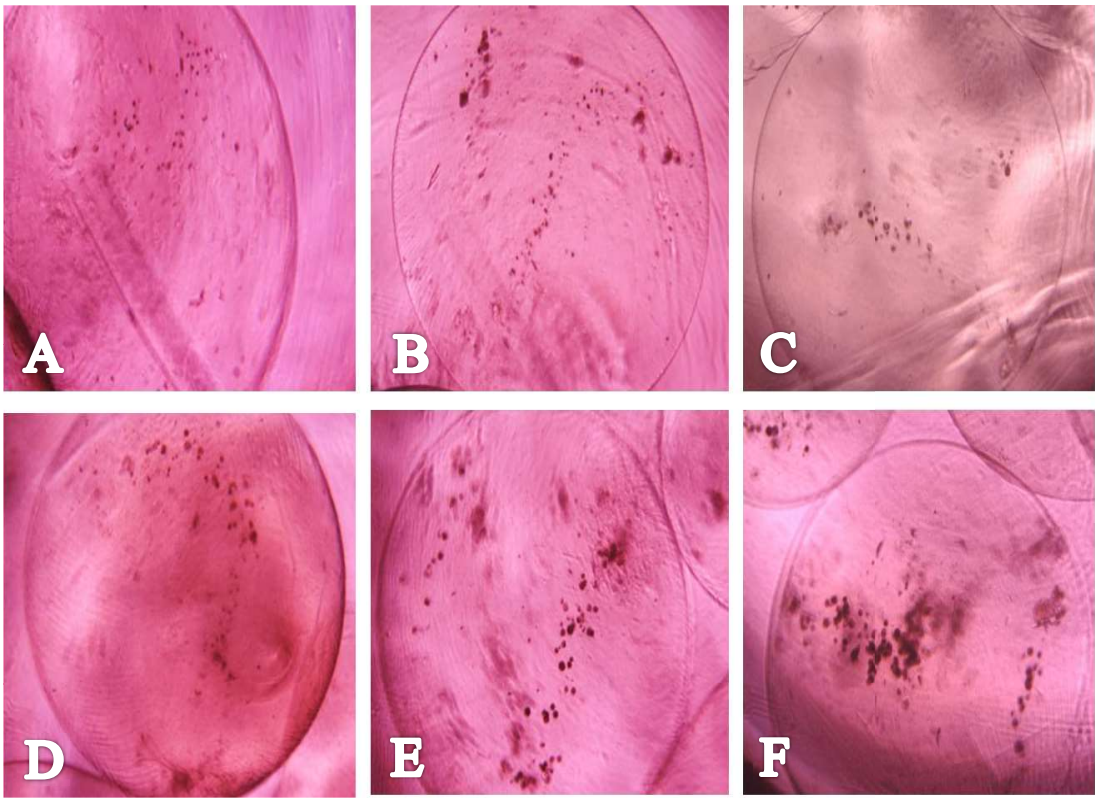

Figure 3.17. Morphology and growth of SK- HEP1 in high glucose 3 days after adding lithium (on day 11 of culture) in: (A) 1.5 percent alginate only, (B) same level alginate +0.05% gelatin, (C) similar amount alginate +0.15% gelatin, (D) normal alginate ratio +0.25% gelatin, (E) exact alginate concentration +0.35% gelatin, (F) similar level alginate +0.45% gelatin.

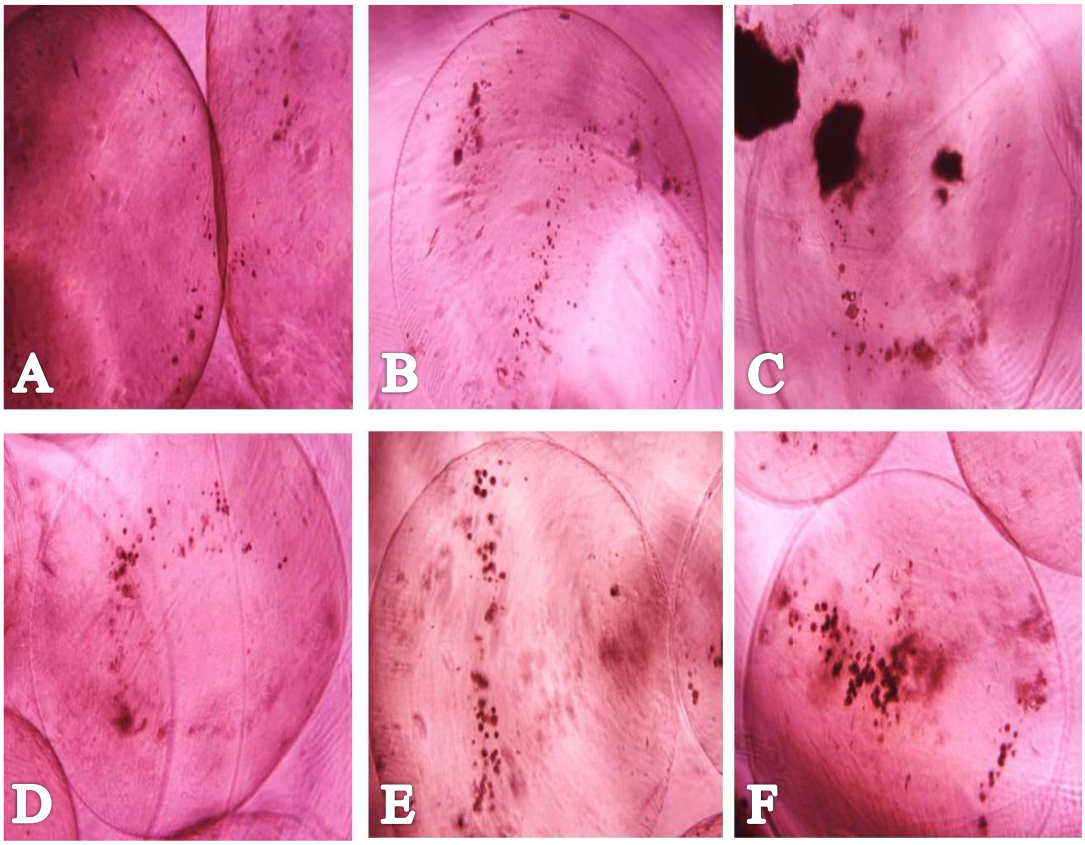

Figure 3.18. Morphology and growth of SK- HEP1 in high glucose 7 days after adding lithium (on day 15 of culture) in: (A) similar previous amount alginate only, (B) similar alginate percentage +0.05% gelatin, (C) normal alginate ratio +0.15% gelatin, (D) same alginate concentration +0.25% gelatin, (E) exact amount alginate +0.35% gelatin, (F) same normal level alginate +0.45% gelatin.

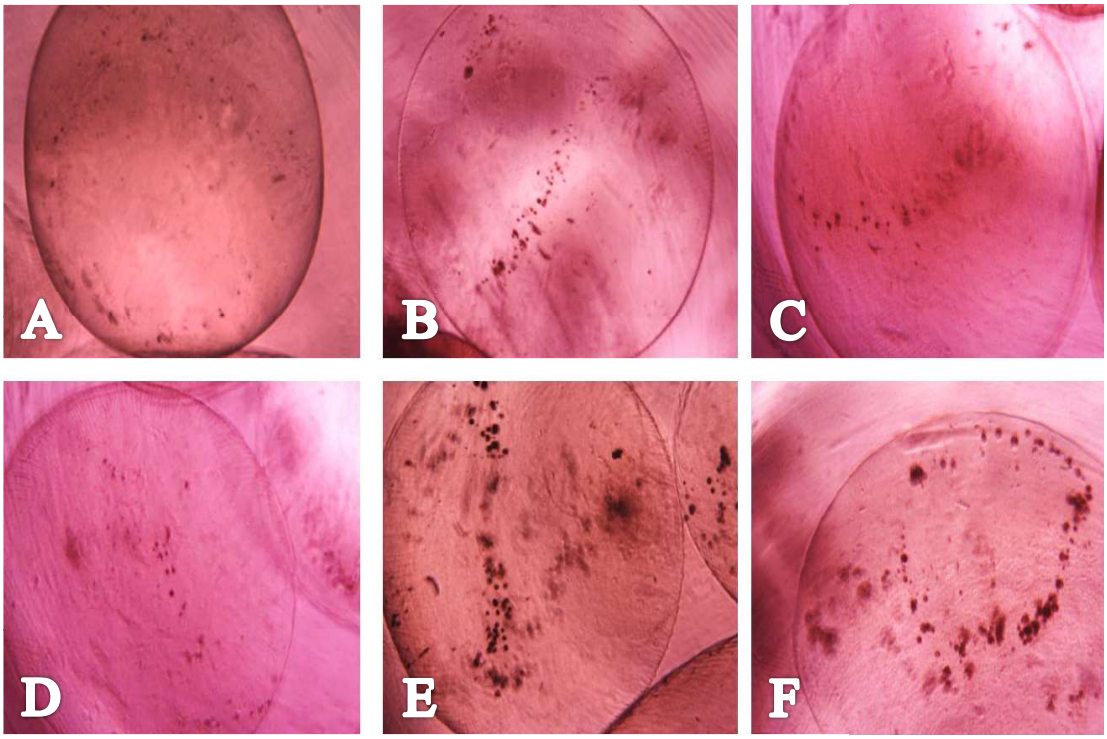

Figure 3.19. Morphology and growth of SK- HEP1 in high glucose 11 days after adding lithium (on day 19 of culture) in: (A) exact previous alginate level only, (B) similar normal alginate amount +0.05% gelatin, (C) same ratio alginate +0.15% gelatin, (D) similar alginate amount+0.25% gelatin, (E) exact alginate concentration +0.35% gelatin, (F) normal level alginate +0.45% gelatin.

3.1.3.2 Spheroids and Lithium Effects in Low Glucose Medium

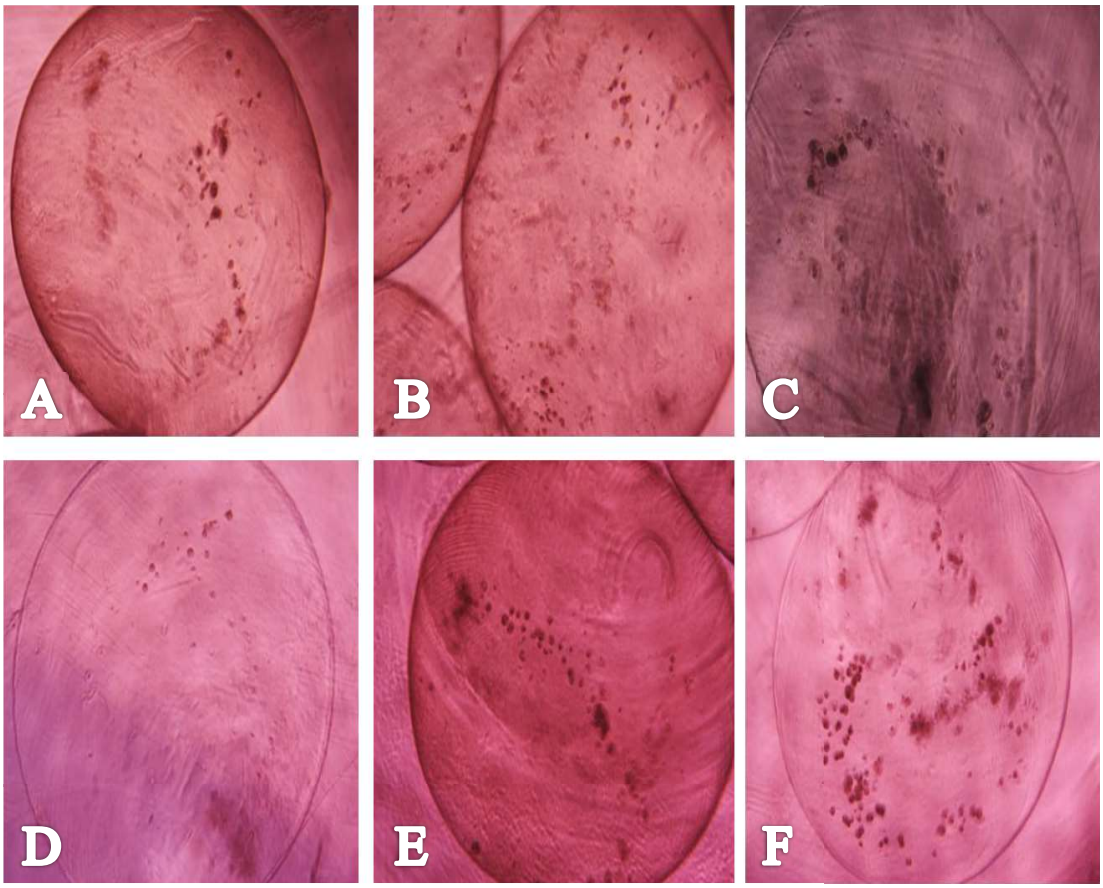

Figure 3.20. Morphology and growth of SK- HEP1 in low glucose 3 days after adding lithium (on day 11 of culture) in: (A) similar previous alginate ratio only, (B) same concentration alginate +0.05% gelatin, (C) exact alginate level +0.15% gelatin, (D) similar amount alginate +0.25% gelatin, (E) same normal ratio alginate +0.35% gelatin, (F) exact alginate amount +0.45% gelatin.

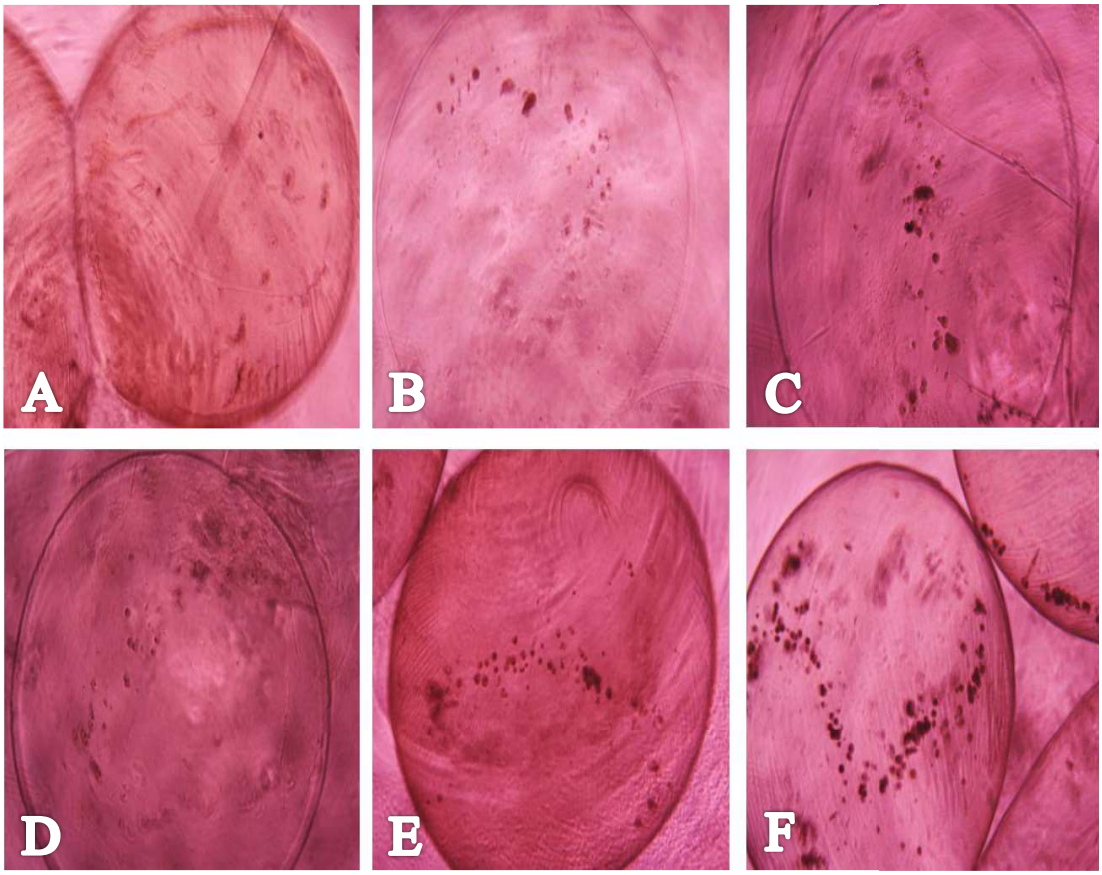

Figure 3.21. Morphology and growth of SK- HEP1 in low glucose 7 days after adding lithium (on day 15 of culture) in: (A) similar alginate amount as normal only, (B) exact same level alginate +0.05% gelatin, (C) similar ratio alginate +0.15% gelatin, (D) same normal concentration alginate +0.25% gelatin, (E) similar alginate percentage +0.35% gelatin, (F) similar normal alginate ratio +0.45% gelatin.

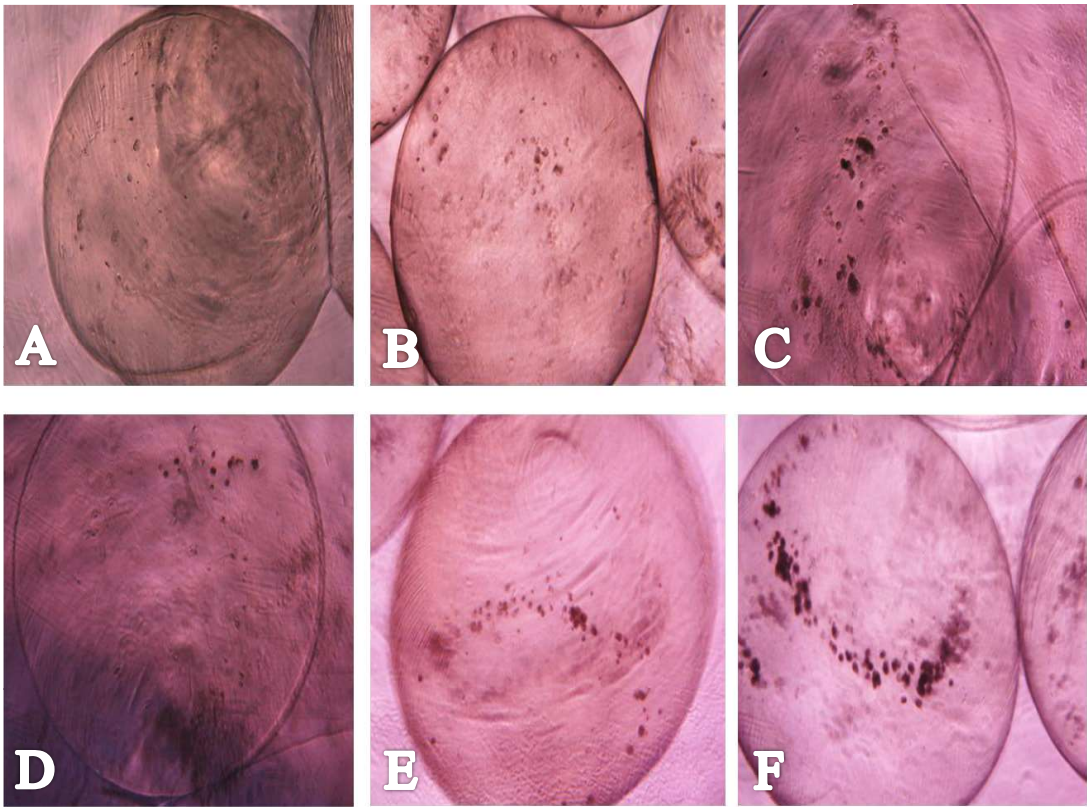

Figure 3.22. Morphology and growth of SK- HEP1 in low glucose 11 days after adding lithium (on day 19 of culture) in: (A) same normal alginate level only, (B) similar ratio alginate +0.05% gelatin, (C) exact alginate amount +0.15% gelatin, (D) similar normal alginate level +0.25% gelatin, (E) exact alginate normal amount+0.35% gelatin, (F) same alginate percentage +0.45% gelatin.

Chapter 4

CONCLUSION

The perfect *in vitro* 3D model of SK- HEP1 ought to give a better illustration of cell behavior *in vivo*. This 3D model of liver adenocarcinoma cells with its capacity to all the more intently copy the *in vivo* tumor conduct, may fill in as an important model for study and use of novel anticancer therapeutics against SK- HEP1.

Alginate- gelatin hydrogels advanced the supplements exchange between cells and the outer environment and allowed cells to proliferate. The hydrogels could not just advance the exchange of supplements and metabolites between the cells and outer condition, in addition, they enabled cells to play out some cell capacities like (proliferation and migration.). Results represented that gelatin had an impact on morphology and supported cell proliferation, but after 1.5% alginate and 0.5% gelatin it disturbed the morphology of the beads. Additionally, the effect of gelatin was clear in both; high and low glucose medium and supported the proliferation at all concentrations.

The ongoing advances in *in vitro* 3D culture innovations, for example, organoids and spheroids, have opened new roads for the advancement of the novel, increasingly physiological human malignant growth models [79, 80]. Medication affectability screening of malignant growth cell lines is routinely performed and enormous scope datasets are publically accessible. In spite of their utility, there are inadequacies

related to customary 2D cell lines which incorporate their inability to reflect tissue/tumor design, a poor achievement the pace of induction, and an absence of related patient neurotic and clinical data [81]. Expenses of new anti-cancer growth drugs have flooded over the previous years due to, among others, the expanding unpredictability of clinical preliminaries and administrative necessities. Meanwhile, the probability that medication will arrive at advertising endorsement in the wake of entering stage 1 clinical testing has continued as before and is essentially lower for hostile to malignant growth drugs contrasted and sedates in other illness regions. While thinking about all signs in oncology, a negligible 1 of every 15 medications that enter clinical advancement will contact US Food what's more, Drug Administration approval [82].

It was obvious that our findings had similarities with information from different studies about the effect of lithium on SK-HEP1 [78]. Nevertheless, instead of culturing them in 2D culture, we cultured them in a 3D system. Lithium had effects to suppress cancer cell proliferation in the 3D system as it successfully made in 2D culture as well.

REFERENCES

[1] R. Yao, R. Zhang, J. Luan, and F. Lin, “Alginate and alginate / gelatin microspheres for human adipose-derived stem cell encapsulation and differentiation,” vol. 025007, 2012, doi: 10.1088/1758-5082/4/2/025007.

[2] M. C. Catoira, L. Fusaro, D. Di, F. Martina, and R. Francesca, “Overview of natural hydrogels for regenerative medicine applications,” *J. Mater. Sci. Mater. Med.*, 2019, doi: 10.1007/s10856-019-6318-7.

[3] X. xi Xu *et al.*, “Encapsulated human hepatocellular carcinoma cells by alginate gel beads as an in vitro metastasis model,” *Exp. Cell Res.*, 2013, doi: 10.1016/j.yexcr.2013.05.013.

[4] S. Haeberle, L. Naegele, R. Burger, F. V. O. N. Stetten, R. Zengerle, and J. Ducre, “Alginate bead fabrication and encapsulation of living cells under centrifugally induced artificial gravity conditions,” vol. 25, no. June, pp. 267–274, 2008, doi: 10.1080/02652040801954333.

[5] M. Science, N. York, and A. Nw, “Designing Cell-Compatible Hydrogels,” vol. 1124, no. 2012, 2013, doi: 10.1126/science.1214804.

[6] V. Kulkarni, K. Butte, and S. Rathod, “Natural Polymers – A Comprehensive Review,” *Int. J. Res. Pharm. Biomed. Sci.*, vol. 3, no. 4, pp. 1597–1613, 2012.

[7] P. De Vos, H. A. Lazarjani, D. Poncelet, and M. M. Faas, “Polymers in cell

encapsulation from an enveloped cell perspective," *Adv. Drug Deliv. Rev.*, vol. 67–68, pp. 15–34, 2014, doi: 10.1016/j.addr.2013.11.005.

[8] S. Rajeswari, "Natural Polymers: a Recent Review," *World J. Pharm. Pharm. Sci.*, vol. 6, no. 8, pp. 472–494, 2017, doi: 10.20959/wjpps20178-9762.

[9] K. Duval *et al.*, "Modeling physiological events in 2D vs. 3D cell culture," *Physiology*, vol. 32, no. 4, pp. 266–277, 2017, doi: 10.1152/physiol.00036.2016.

[10] S. Afewerki, A. Sheikhi, S. Kannan, S. Ahadian, and A. Khademhosseini, "Gelatin-polysaccharide composite scaffolds for 3D cell culture and tissue engineering: Towards natural therapeutics," *Bioeng. Transl. Med.*, 2019, doi: 10.1002/btm2.10124.

[11] B. Sarker *et al.*, "Fabrication of alginate-gelatin crosslinked hydrogel microcapsules and evaluation of the microstructure and physico-chemical properties," *J. Mater. Chem. B*, vol. 2, no. 11, pp. 1470–1482, 2014, doi: 10.1039/c3tb21509a.

[12] M. Szekalska, A. Puciłowska, E. Szymańska, P. Ciosek, and K. Winnicka, "Alginate: Current Use and Future Perspectives in Pharmaceutical and Biomedical Applications," *Int. J. Polym. Sci.*, vol. 2016, 2016, doi: 10.1155/2016/7697031.

[13] S. Chowdhury, I. R. Chowdhury, F. Kabir, M. A. J. Mazumder, M. H. Zahir,

and K. Alhooshani, “Alginate-based biotechnology: A review on the arsenic removal technologies and future possibilities,” *J. Water Supply Res. Technol. - AQUA*, vol. 68, no. 6, pp. 369–389, 2019, doi: 10.2166/aqua.2019.005.

[14] W. H. Tan and S. Takeuchi, “Monodisperse alginate hydrogel microbeads for cell encapsulation,” *Adv. Mater.*, vol. 19, no. 18, pp. 2696–2701, 2007, doi: 10.1002/adma.200700433.

[15] V. Nedovic, “Carrier for cell immobilisation jan egil melvik and michael dornish,” pp. 33–51, 2004.

[16] L. Gasperini, J. F. Mano, and R. L. Reis, “Natural polymers for the microencapsulation of cells,” *J. R. Soc. Interface*, vol. 11, no. 100, 2014, doi: 10.1098/rsif.2014.0817.

[17] K. I. Draget and C. Taylor, “Chemical, physical and biological properties of alginates and their biomedical implications,” *Food Hydrocoll.*, vol. 25, no. 2, pp. 251–256, 2011, doi: 10.1016/j.foodhyd.2009.10.007.

[18] S. J. Bidarra, C. C. Barrias, and P. L. Granja, “Injectable alginate hydrogels for cell delivery in tissue engineering,” *Acta Biomater.*, vol. 10, no. 4, pp. 1646–1662, 2014, doi: 10.1016/j.actbio.2013.12.006.

[19] A. S. Hoffman, “Hydrogels for biomedical applications,” *Adv. Drug Deliv. Rev.*, vol. 64, no. SUPPL., pp. 18–23, 2012, doi: 10.1016/j.addr.2012.09.010.

[20] C. K. Kuo and P. X. Ma, “Ionically crosslinked alginate hydrogels as scaffolds for tissue engineering: Part 1. Structure, gelation rate and mechanical properties,” *Biomaterials*, vol. 22, no. 6, pp. 511–521, 2001, doi: 10.1016/S0142-9612(00)00201-5.

[21] S. N. Pawar and K. J. Edgar, “Biomaterials Alginate derivatization : A review of chemistry , properties and applications,” *Biomaterials*, vol. 33, no. 11, pp. 3279–3305, 2012, doi: 10.1016/j.biomaterials.2012.01.007.

[22] A. Khavari, M. Nydén, D. A. Weitz, and A. J. Ehrlicher, “Composite alginate gels for tunable cellular microenvironment mechanics,” *Sci. Rep.*, vol. 6, no. August, pp. 1–11, 2016, doi: 10.1038/srep30854.

[23] F. Ozawa *et al.*, “Alginate gel microwell arrays using electrodeposition for three-dimensional cell culture,” *Lab Chip*, vol. 13, no. 15, pp. 3128–3135, 2013, doi: 10.1039/c3lc50455g.

[24] V. Iansante *et al.*, “A new high throughput screening platform for cell encapsulation in alginate hydrogel shows improved hepatocyte functions by mesenchymal stromal cells co-encapsulation,” *Front. Med.*, 2018, doi: 10.3389/fmed.2018.00216.

[25] E. Boanini and A. Bigi, “Biomimetic gelatin-octacalcium phosphate core-shell microspheres,” *J. Colloid Interface Sci.*, vol. 362, no. 2, pp. 594–599, 2011, doi: 10.1016/j.jcis.2011.06.061.

[26] P. Jaipan, A. Nguyen, and R. J. Narayan, “Gelatin-based hydrogels for biomedical applications,” *MRS Commun.*, 2017, doi: 10.1557/mrc.2017.92.

[27] J. Coyne, N. Zhao, A. Olubode, M. Menon, and Y. Wang, “Development of hydrogel-like biomaterials via nanoparticle assembly and solid-hydrogel transformation,” *J. Control. Release*, vol. 318, pp. 185–196, 2020, doi: 10.1016/j.jconrel.2019.12.026.

[28] M. Xu, L. Wei, Y. Xiao, H. Bi, H. Yang, and Y. Du, “Physicochemical and functional properties of gelatin extracted from Yak skin,” *Int. J. Biol. Macromol.*, vol. 95, pp. 1246–1253, 2017, doi: 10.1016/j.ijbiomac.2016.11.020.

[29] J. W. Nichol, S. T. Koshy, H. Bae, C. M. Hwang, S. Yamanlar, and A. Khademhosseini, “Cell-laden microengineered gelatin methacrylate hydrogels,” *Biomaterials*, vol. 31, no. 21, pp. 5536–5544, 2010, doi: 10.1016/j.biomaterials.2010.03.064.

[30] K. Pal, A. K. Banthia, and D. K. Majumdar, “Preparation and characterization of polyvinyl alcohol-gelatin hydrogel membranes for biomedical applications,” *AAPS PharmSciTech*, vol. 8, no. 1, 2007, doi: 10.1208/pt080121.

[31] R. Dash, M. Foston, and A. J. Ragauskas, “Improving the mechanical and thermal properties of gelatin hydrogels cross-linked by cellulose nanowhiskers,” *Carbohydr. Polym.*, vol. 91, no. 2, pp. 638–645, 2013, doi: 10.1016/j.carbpol.2012.08.080.

[32] Z. Fan, P. Cheng, G. Yin, Z. Wang, and J. Han, “In situ forming oxidized salecan/gelatin injectable hydrogels for vancomycin delivery and 3D cell culture,” *J. Biomater. Sci. Polym. Ed.*, vol. 0, no. 0, p. 000, 2020, doi: 10.1080/09205063.2020.1717739.

[33] P. Dubruel *et al.*, “Porous Gelatin Hydrogels : 2 . In Vitro Cell Interaction Study,” vol. 2959, pp. 338–344, 2007.

[34] E. Boanini, K. Rubini, S. Panzavolta, and A. Bigi, “Acta Biomaterialia Chemico-physical characterization of gelatin films modified with oxidized alginate,” *Acta Biomater.*, vol. 6, no. 2, pp. 383–388, 2010, doi: 10.1016/j.actbio.2009.06.015.

[35] J. F. Mano *et al.*, “Natural origin biodegradable systems in tissue engineering and regenerative medicine : present status and some moving trends Natural origin biodegradable systems in tissue engineering and regenerative medicine : present status and some moving trends,” 2007, doi: 10.1098/rsif.2007.0220.

[36] K. Deshmukh, M. B. Ahamed, R. R. Deshmukh, S. K. K. Pasha, P. R. Bhagat, and K. Chidambaram, *3 - Biopolymer Composites With High Dielectric Performance: Interface Engineering*. Elsevier Inc., 2017.

[37] S. R. Caliari and J. A. Burdick, “HHS Public Access,” vol. 13, no. 5, pp. 405–414, 2018, doi: 10.1038/nmeth.3839.A.

[38] A. Manuscript, “NIH Public Access,” vol. 8, no. 5, pp. 607–626, 2012, doi:

10.1586/erd.11.27.Design.

[39] M. P. Lutolf, “Biomaterials: Spotlight on hydrogels,” *Nat. Mater.*, vol. 8, no. 6, pp. 451–453, 2009, doi: 10.1038/nmat2458.

[40] K. Y. Lee and D. J. Mooney, “Hydrogels for tissue engineering,” *Chem. Rev.*, vol. 101, no. 7, pp. 1869–1879, 2001, doi: 10.1021/cr000108x.

[41] A. Roy, A. K. Bajpai, and J. Bajpai, “Designing swellable beads of alginate and gelatin for controlled release of pesticide (cypermethrin),” *J. Macromol. Sci. Part A Pure Appl. Chem.*, vol. 46, no. 9, pp. 847–859, 2009, doi: 10.1080/10601320903077976.

[42] K. Yue, G. Trujillo-de Santiago, M. M. Alvarez, A. Tamayol, N. Annabi, and A. Khademhosseini, “Synthesis, properties, and biomedical applications of gelatin methacryloyl (GelMA) hydrogels,” *Biomaterials*, vol. 73, pp. 254–271, 2015, doi: 10.1016/j.biomaterials.2015.08.045.

[43] Y. Du *et al.*, “A Spray-Filming Self-Healing Hydrogel Fabricated from Modified Sodium Alginate and Gelatin as a Bacterial Barrier,” *Macromol. Biosci.*, vol. 20, no. 2, pp. 1–11, 2020, doi: 10.1002/mabi.201900303.

[44] H. Takahashi *et al.*, “Anticancer polymers designed for killing dormant prostate cancer cells,” *Sci. Rep.*, vol. 9, no. 1, pp. 1–11, 2019, doi: 10.1038/s41598-018-36608-5.

[45] T. Andersen, P. Auk-Emblem, and M. Dornish, “3D Cell Culture in Alginate Hydrogels,” *Microarrays*, 2015, doi: 10.3390/microarrays4020133.

[46] G. D. Nicodemus and S. J. Bryant, “Cell encapsulation in biodegradable hydrogels for tissue engineering applications,” *Tissue Eng. - Part B Rev.*, vol. 14, no. 2, pp. 149–165, 2008, doi: 10.1089/ten.teb.2007.0332.

[47] A. Belscak-Cvitanovic *et al.*, “Improving the controlled delivery formulations of caffeine in alginate hydrogel beads combined with pectin, carrageenan, chitosan and psyllium,” *Food Chem.*, vol. 167, pp. 378–386, 2015, doi: 10.1016/j.foodchem.2014.07.011.

[48] G. Simó, E. Fernández- Fernández, J. Vila- Crespo, V. Ruipérez, and J. M. Rodríguez- Nogales, “Research progress in coating techniques of alginate gel polymer for cell encapsulation,” *Carbohydr. Polym.*, vol. 170, pp. 1–14, 2017, doi: 10.1016/j.carbpol.2017.04.013.

[49] C. A. Hoesli *et al.*, “Mammalian cell encapsulation in alginate beads using a simple stirred vessel,” *J. Vis. Exp.*, vol. 2017, no. 124, 2017, doi: 10.3791/55280.

[50] Wu, “Biomedical and Pharmaceutical Application of Fish Collagen and Gelatin: A Review,” *J. Nutr. Ther.*, no. February 2014, 2013, doi: 10.6000/1929-5634.2013.02.04.6.

[51] G. Buhus, C. Peptu, M. Popa, and J. Desbrières, “Controlled release of water

soluble antibiotics by carboxymethylcellulose- And gelatin-based hydrogels crosslinked with epichlorohydrin," *Cellul. Chem. Technol.*, vol. 43, no. 4–6, pp. 141–151, 2009.

[52] O. O. Oyeleye, S. T. Ogundeji, S. I. Ola, and O. G. Omitogun, "Basics of animal cell culture : Foundation for modern science," vol. 11, no. May, pp. 6–16, 2016, doi: 10.5897/BMBR2016.0261.

[53] *CELL CULTURE BASICS Handbook*.

[54] D. Anton, H. Burckel, E. Josset, and G. Noel, "Three-dimensional cell culture: A breakthrough in vivo," *Int. J. Mol. Sci.*, vol. 16, no. 3, pp. 5517–5527, 2015, doi: 10.3390/ijms16035517.

[55] A. I. Khoruzhenko, "2D- and 3D-cell culture," *Biopolym. Cell*, vol. 27, no. 1, pp. 17–24, 2011, doi: 10.7124/bc.00007D.

[56] M. Kapałczyńska *et al.*, "2D and 3D cell cultures – a comparison of different types of cancer cell cultures," *Arch. Med. Sci.*, vol. 14, no. 4, pp. 910–919, 2018, doi: 10.5114/aoms.2016.63743.

[57] S. Bhatia and S. Bhatia, "Introduction to animal tissue culture science," *Introd. to Pharm. Biotechnol. Vol. 3*, 2019, doi: 10.1088/2053-2563/aafac0ch1.

[58] C. Biosensors, R. Edmondson, J. J. Broglie, A. F. Adcock, and L. Yang, "Three-Dimensional Cell Culture Systems and Their Applications in Drug Discovery

and Cell-Based Biosensors," vol. 12, no. 4, pp. 207–218, 2014, doi: 10.1089/adt.2014.573.

[59] V. Guduric, *Chapter 7 - Membrane scaffolds for 3D cell culture*. 2020.

[60] S. Chen, Y. Chang, S. Nieh, C. Liu, C. Yang, and Y. Lin, "Nonadhesive Culture System as a Model of Rapid Sphere Formation with Cancer Stem Cell Properties," vol. 7, no. 2, 2012, doi: 10.1371/journal.pone.0031864.

[61] S. Khetan and J. A. Burdick, "Biomaterials Patterning network structure to spatially control cellular remodeling and stem cell fate within 3-dimensional hydrogels," *Biomaterials*, vol. 31, no. 32, pp. 8228–8234, 2010, doi: 10.1016/j.biomaterials.2010.07.035.

[62] L. G. Griffith and M. A. Swartz, "Capturing complex 3D tissue physiology in vitro," vol. 7, no. March, pp. 211–224, 2006, doi: 10.1038/nrm1858.

[63] J. A. Hickman *et al.*, "Three-dimensional models of cancer for pharmacology and cancer cell biology : Capturing tumor complexity in vitro / ex vivo," pp. 1115–1128, 2014, doi: 10.1002/biot.201300492.

[64] K. M. Yamada and E. Cukierman, "Review Modeling Tissue Morphogenesis and Cancer in 3D," pp. 601–610, 2007, doi: 10.1016/j.cell.2007.08.006.

[65] T. Mseka, J. R. Bamburg, and L. P. Cramer, "ADF / cofilin family proteins control formation of oriented actin-filament bundles in the cell body to trigger

fibroblast polarization," pp. 4332–4344, 2007, doi: 10.1242/jcs.017640.

[66] S. Breslin and L. O. Driscoll, "Three-dimensional cell culture : the missing link in drug discovery," *Drug Discov. Today*, vol. 18, no. 5–6, pp. 240–249, 2013, doi: 10.1016/j.drudis.2012.10.003.

[67] B. B. Aggarwal, D. Danda, S. Gupta, and P. Gehlot, "Models for prevention and treatment of cancer : Problems vs promises," vol. 78, pp. 1083–1094, 2009, doi: 10.1016/j.bcp.2009.05.027.

[68] L. Weiswald, D. Bellet, and V. Dangles-marie, "Spherical Cancer Models in Tumor," *NEO*, vol. 17, no. 1, pp. 1–15, 2015, doi: 10.1016/j.neo.2014.12.004.

[69] R. D. Puga, D. Antonow, P. Severino, and C. Bonorino, "Comparison of 2D and 3D cell culture models for cell growth, gene expression and drug resistance," *Mater. Sci. Eng. C*, p. 110264, 2019, doi: 10.1016/j.msec.2019.110264.

[70] M. Fane and A. T. Weeraratna, "How the ageing microenvironment influences tumour progression," *Nat. Rev. Cancer*, doi: 10.1038/s41568-019-0222-9.

[71] J. W. Haycock, "Chapter 1 3D Cell Culture : A Review of Current Approaches and Techniques," no. January 2011, pp. 0–15, 2015, doi: 10.1007/978-1-60761-984-0.

[72] A. Alizadeh, L. Rezakhani, M. Anjom, and S. Ghasemi, "Tissue and Cell Frequency of CD44 positive cells in MKN45 cell line after treatment with

docetaxel in two and three-dimensional cell cultures," *Tissue Cell*, vol. 63, no. September 2019, p. 101324, 2020, doi: 10.1016/j.tice.2019.101324.

[73] B. Larson, S. A. Scientist, and B. Instruments, "h i t e a p e r 3D Cell Culture : A Review of Current Techniques," vol. 6, no. Figure 1, pp. 1–10, 2015.

[74] S. Communication, "i l n e n s r i t i t r e l c i l s t," vol. 52, no. October 2019, pp. 1–4, 2020.

[75] E. Bresnahan, P. Ramadori, M. Heikenwalder, and L. Zender, "Novel patient-derived preclinical models of liver cancer," *J. Hepatol.*, vol. 72, no. 2, pp. 239–249, 2020, doi: 10.1016/j.jhep.2019.09.028.

[76] P. De Vos and R. Van Schilfgaarde, "Biocompatibility Issues," 1999.

[77] A. Zuchowska, A. Buta, B. Dabrowski, E. Jastrzebska, K. Zukowski, and Z. Brzozka, "l P of," *Sensors Actuators B. Chem.*, p. 127064, 2019, doi: 10.1016/j.snb.2019.127064.

[78] E. Erdal, N. Ozturk, T. Cagatay, E. Eksioglu-Demiralp, and M. Ozturk, "Lithium-mediated downregulation of PKB/Akt and cyclin E with growth inhibition in hepatocellular carcinoma cells," *Int. J. Cancer*, vol. 115, no. 6, pp. 903–910, 2005, doi: 10.1002/ijc.20972.

[79] J. Drost and H. Clevers, "Organoids in cancer research," *Nat. Rev. Cancer*, doi: 10.1038/s41568-018-0007-6.

[80] J. Kriston-vizi and H. Flotow, “Getting the Whole Picture : High Content Screening Using Three-Dimensional Cellular Model Systems and Whole Animal Assays,” pp. 1–8, 2016, doi: 10.1002/cyto.a.22907.

[81] H. E. Francies, A. Barthorpe, A. Mclaren-douglas, W. J. Barendt, and M. J. Garnett, “Drug Sensitivity Assays of Human Cancer Organoid Cultures,” 2016, doi: 10.1007/7651.

[82] F. Weeber, S. N. Ooft, K. K. Dijkstra, and E. E. Voest, “Review Tumor Organoids as a Pre-clinical Cancer Model for Drug Discovery,” *Cell Chem. Biol.*, pp. 1–9, 2017, doi: 10.1016/j.chembiol.2017.06.012.