3D Hydrogel Systems

Supporting the Growth of Neural Stem Cells

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Abstract

Stem cell-based therapy holds a great promise for the treatment of central nervous system (CNS) injuries/diseases. Successful regeneration of damaged neural tissue is based on big amount of transplanted cells. The low viability of transplanted cells and the limited differentiation toward specific cell types make the required number of cells per treatment even much higher. The existing cell culture system cannot provide fast proliferation of neural stem cells (NSCs) for clinical treatment. Therefore, developing three-dimensional (3D) cell culture systems by mimicking the niche of NSCs to support their growth in vitro is required by clinical treatment. Among the components of niche, extracellular matrix, with its specific biochemical, biomechanical and physical properties, play an essential role in cell activities by direct or indirect interactions with the cells. Matrices with 3D architectures are widely investigated, which are providing the needed support for emerging applications in tissue engineering and stem cell research. Many substances have been used to fabricate matrices, hydrogel is getting more popular due to its similar mechanical properties as the natural matrix and providing suitable environment for neural stem cells. Thus, the creation of a 3D hydrogel culture system, which can provide controlled gelation time, uniform suspension of cells, biophysical and biochemical properties that is favorable for NSCs survival and growth as well as enhancing cell proliferation, is desirable for clinical treatment. In this paper, hydrogel of pure gellan gum and hybrid hydrogels of the gellan gum and konjac gum were studied. The stiffness of gels was tested by rheometers and suitable gels were used for cell culture. Cell culture of NSCs showed that the hydrogels of gellan gum or gellan/konjac hybrid gels can support NSCs growth and proliferation, the most proliferation and viability appeared in the1.4% gellan gum (stiffness 250Pa) and many neurospheres formed in the 1.1% gellan gum based hybrid gels (constant 1.1% gellan gum and various konjac concentration). The cultures of mesenchymal stem cell (MSCs) and meningeal fibroblast isolated from brain tissue revealed that different cell type prefer different gels with different composition and concentration.

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List of Abbreviations

2D	Two dimensional
3D	Three dimensional
bFGF	Basic fibroblast growth factor
CAM	Cell adhesion molecules
CNS	Central nervous system
CSPG	Chondroitin sulfate proteoglycans
DMEM	Dulbecco's modified eagle's medium
DSC	Differential scanning calorimetry
EC	Endothelial cells
ECM	Extracellular matrix
EDC	1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride
EDTA	Ethylenediaminetetraacetic acid
EGF	Epidermal growth factor
ES	Embryonic stem cells
FBS	Fetal bovine serum
GG	Gellan gum
HA	Hyaluronic acid
IKVAV	Ile-Lys-Val-Ala-Val sequence
IPSC	Induced pluripotent stem cells
KGM	Konjac glucomannan
LVR	Linear viscoelastic region
MAC	Methacrylamide chitosan
MF	Meningeal fibroblast
MSC	Mesenchymal stromal cells
MSCGS	Mesenchymal stem cell growth supplement serum free
NCFC	Neural Colony-Forming Cell
NEP	Neuroepithelial progenitors
NHS	N-hydroxysulfoxuccinimide
NPC	Meural progenitor cell
NSA	Neurosphere assay
NSC	Neural stem cells
NSPC	Neural stem/progenitor cells
OAS	Oscillation amplitude sweep
OFS	Oscillation frequency sweep
OPC	Oligodendrocyte precursor cells
Pa	Pascal
PBS	Phosphate-buffered saline
PEG	Poly(ethylene glycol)
PF127	Pluronic F127 (generic name, poloxamer 407)
PLA	Polylactide
PLGA	Poly(lactic-co-glycolic acid)
RGD	Arg-Gly-Asp sequence
SVZ	Subventricular zone
TNC	Tenascin C
UV	Ultraviolet
VCU	Virginia Commonwealth University

1 Introduction

The central nervous system (CNS) comprises of the brain and spinal cord. It plays the central role in the control of most body functions. The damage of CNS can be caused by either injury of brain or spinal cord or the neurological disorders, as well as cancer. The common pathophysiological change resulting from the damage is the death of neuron (nerve cell), broken axons with demyelination, loss of the glia support, and the change of environment of the injured site in CNS. Currently the clinical treatments try to manage the primary injury and reduce the secondary injury. But none is available to regenerate the damaged CNS tissue and restore the impacted function since the CNS has limited capacity to regenerate and replace the damaged cells lost during the injury or disease [1, 2]. Therefore, a clinical treatment for repairing the damaged CNS and restoring the function is being investigated. The transplantation of neural stem cells (NSCs) has appealing potential for regenerating the damaged CNS. However, NSCs transplantation therapy faces the problem of the fate of transplanted cells which is the big obstacle for its further clinical use [3]. Large numbers of functional cells that differentiated from stem cells is needed to achieve the effect of treatment and the low viability of transplanted cells in vivo make this required number even higher. For example, 5 million dopaminergic neurons per transplantation is required to treat Parkinson`s Disease. The existing cell culture system can`t provide fast proliferation of NSCs for clinical treatment. Therefore, to enhance the cell viability and proliferation in vitro becomes the key point for stem cell therapy since both cell viability and proliferation correlate to functional outcome directly.

1.1 Neural Stem Cells (NSCs)

1.1.1 Neural Stem Cells Characteristic

Neural stem cells (NSCs) are multi-potent populations present in both fetal and adult brain, they are capable of self-renew and have the ability to differentiate into three major neural cells (neurons, astrocytes and oligodendrocytes) (Figure 1) through asymmetrical fate-committed division [4, 5]. Although there exist neural progenitors which refers to a cell with a more restricted potential than a stem cell, but in this paper the NSCs are used to refer to all multi-potent stem cells.

NSCs exist through the whole life and tree main types of NSCs have been identified depending on the time of development [6]. First, NSCs exist as

neuroepithelial progenitors (NEP) in the earliest stage of development to be responsible to form the neural plate, which continue to form the neural tube. Later, NSCs exist as radial glia (RG) cells, which originate from NEP but have more limited differentiation potential even some of them are capable to differentiate to neuronal, glial and oligodendroglial cells. Finally, the NSCs were identified in adult brain in the subventricular zone (SVZ) [7], the dentate gyrus of the hippocampus [8] and external germinal layer of cerebellum [4].The SVZ is the region in the adult brain with the highest neurogenetic rate, and NSCs isolated from SVZ showed the ability to give rise to neural cells [9] and non-neural cells [10, 11]. NSCs have been isolated also from non-neurogenic areas, such as the spinal cord [12]. In vitro the NSCs showed the stability after multiple passages without loss of their multipotentiality [13]. Thus, NSCs give a promising potential of cell therapy for the damaged CNS.

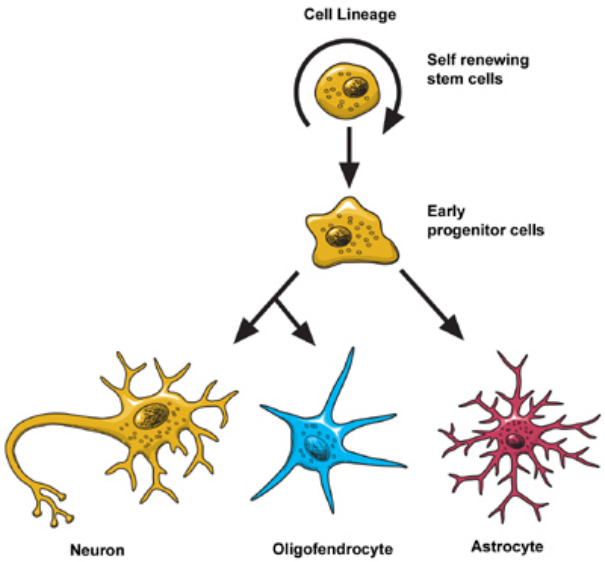


Figure 1: Stem cells differentiate to produce different types of nerve cells [14]

1.1.2 Neural Stem Cells for CNS Regeneration

NSCs therapy offers highly attractive potential for the regeneration of injured CNS, such as replacement of damaged cells, promotion of axon regrowth, re-myelination of damaged axons and rebuilding of neuronal circuitry [15]. This can be done either by transplantation of exogenous NSCs or by stimulation of endogenous NSCs. Stimulation of endogenous NSCs is through the neurogenesis in adult brain due to the proliferation and differentiation of stem cells which resides in the adult CNS [9, 16]. Cell populations generated from endogenous stimulation may not be sufficient to replace damaged or lost tissue in CNS injury, especially for the areas with little evidence of ongoing neurogenesis like spinal cord and

striatum. Transplantation of exogenous NSCs can be achieved by transplanting in vitro expanded stem cells and/or their neuronal progeny, either by directly replacing the damaged cells or indirectly by secreting trophic factors to promote endogenous regeneration. NSCs transplantation aims not only at substituting dead cells with newly differentiated neural cells but also at promoting functional integration into neural circuits of newly formed cells. A variety of different cell sources for cell transplantation have been investigated and tested. They can be either isolated from neural tissue, such as fetal or adult CNS-derived NSCs [4], or derived from non-neural stem cells, such as mesenchymal stem cells (MSCs) [17], directed differentiation of embryonic stem cells (ES) [18], and from induced pluripotent stem cells (IPSC) [19].

Many investigations have been done in vitro and in vivo for NSCs transplantation. Experiments showed that NSCs cultured in vitro could give rise to functional neurons that are connected and electrically active upon transplantation into brain of rats [20]. The immunomodulatory properties of NSCs have also been investigated in vitro [21]. The rodent models, such as inflammatory diseases like multiple sclerosis [22], spinal cord lesions [23] and neurodegenerative disorder [24-26] have been used to demonstrate cell therapy. Purified human neural stem cells generated from an aborted human fetal brain for clinical trial in Pelizaeus–Merzbacher disease [27] showed that the 2-year follow-up report indicated safety and improved long-term myelination, and the cells are on-going in phase I/II clinical trials for spinal cord injury [28].

For the goal of cell therapy, the implanted NSCs must be able to survive and proliferate to a required population for secreting trophic factors to support endogenous neurogenesis or replace damaged tissue. They need to be able to differentiate into the appropriate types that functionally integrate into the damaged host brain circuitry [29]. Therapeutic effects of transplanted NSCs have been evaluated in different ways. Integration and reconstruction of the neuronal circuitry, which needs big amount of cells, is an important goal for restoring the damaged CNS function. However, the fate of transplanted cells is strongly influenced by the type of diseases/injuries of the CNS and the biomechanical and/or biochemical signals in the microenvironment [30]. Low viability and survival rate of transplanted stem cells hinder the further clinical use. Thus, the cell viability and proliferation becomes the key point for cell therapy since both correlate to the functional

outcome directly. For the clinical treatment a big amount of desired NSCs with natural phonotype and genotype is needed. Existing cell culture systems can`t meet this requirement. Therefore, developing better cell culture systems is necessary.

1.2 NSCs Cell Culture System

1.2.1 Current NSCs Cell Culture System

Currently there are two NSCs culture systems commonly used, the neurosphere assay (NSA) and adhesive culture in 2D. In the NSA the micro-dissected neural tissues were dissociated and plated on low-attachment culture flasks in serum-free media in presence of mitogen (EGF and bFGF). NSCs respond to mitogens and begin to proliferate after 24hours and form cluster by 2-3 days. The clusters detach after 3-5 days culture and float in media. Mostly by day 7 neurospheres with 100–200 μ m in diameter which composed of approximately 10,000 cells can be obtained. This is called primary neurospheres that can be dissociated and re-plated to generate neurospheres (Figure 2 and Figure 3) [31]. This procedure can be sequentially repeated several times to expand a NSC population. The cells from neurospheres derived from embryonic mouse CNS tissue treated in this manner have been passaged for up to 10 weeks with no loss in their proliferative ability, resulting in a 10^7 fold increase in total cell number [31]. But NSA has its limit. Both neural stem and progenitor cells were isolated and expanded by NSA. NSA does not provide a reliable or accurate readout of stem cell frequency [32]. Neurospheres and neurosphere-forming cells are morphologically and functionally heterogeneous [33]. Neural Colony-Forming Cell (NCFC) assay was investigated which is able to better discriminate between neural stem and progenitor cells compared to the neurosphere assay [31]. Another method is the adhesive 2D culture, in which NSCs grow on coated substrates to form a monolayer (Figure 4). Laminin is the commonly used coating substrate [34, 35], and the mostly used growth factors to maintain self-renewal of cultured adult NSCs are epidermal growth factor (EGF) and basic fibroblast growth factor (FGF) [36]. NSCs can be competently expanded as adherent clonal homogeneous NSC lines and cells divide symmetrically, retaining their tripotency differentiation capacity. Compared to the neurosphere assay, adherent culture is more easily monitored and has better access to growth factors [37]. It have been shown that 2D cultures with less

differentiated cells than neurosphere assay, in which cell–cell contacts and non-uniform mitogens may stimulate differentiation programs [6].

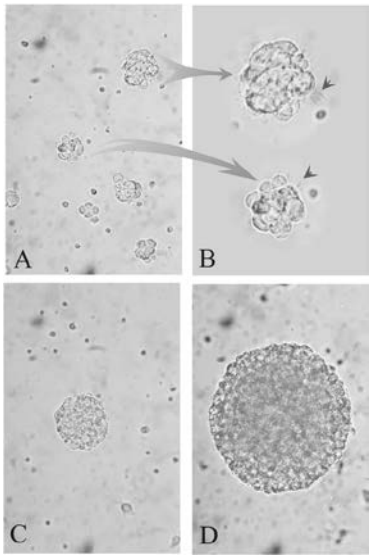


Figure 2 : Neurosphere assay
(A) Cluster formed after 2 days subpassing of cells isolated from cortex. (B) Healthy neurospheres appearance microspikes (arrow heads) on enlarged spheres from A. (C) By 4 days in vitro neurospheres have grown in size, detached from the substrate and floated in suspension. (D) A floating 7 days neurosphere. Magnification: A B D ×200; B ×400.

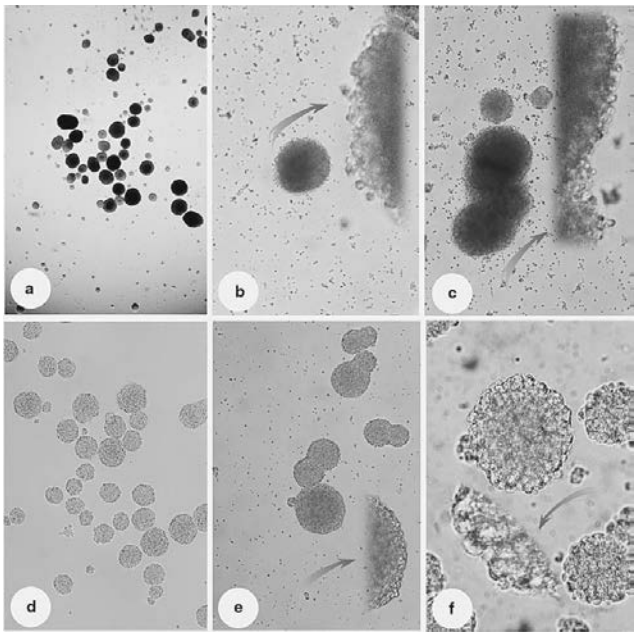


Figure 3: Healthy (d–f) and unhealthy (a–c) neurospheres
Dark in the center is a sign that a number of cells are dying. Light in color and translucent means that it is composed live healthy cells. The inset (arrow) represents a higher magnification of the right side of sphere which showed that the center of the sphere is becoming dark but the outer portion of the sphere is still light and translucent.

Even through, it has shown that cells often exhibit unnatural behavior when they are excised from native three dimensional (3D) tissues and confined to a monolayer. Comparing to the complexities of real tissue physiology, the 2D culture environment is lack of spatial organization, which can influence cell phenotype, genotype, morphology, migration, cell attachment and other bioprocesses (Table 1). In native tissue, cells live in a 3D dynamic microenvironment which can regulate cell fate through either cell–cell or cell–matrix interaction. This cellular microenvironment contributes to the spatially and temporally complex signaling domain that directs cell phenotype. Based on Bissell's concept, the phenotype can supersede genotype simply due to interactions with the ECM. Cells are not only defined by its genome, but also evaluated in the context of the ECM and other factors in their environment, which orchestrates an intracellular signaling cascade that influences phenotypic fate by altering gene and ultimately protein expression

[38]. In a 2D environment, cells can alter production of their own ECM proteins and undergo morphological changes [39], and alter metabolism as well as their gene expression. Further, in a 2D culture it is not possible to create molecular gradients which play a vital role in differentiation, cell fate, signal transduction, neural information transmission and other biological processes. All these can result in the influence on the movements of cells which normally follow a chemical signal or molecular gradient in nature tissue [39].

In 2D culture system, only part of cell membrane attaches to the ECM or interact with the neighbor cells, the rest part expose to the culture media. This unnatural intergrin binding affect intracellular signaling and cell cluster in the correct way in turn to affect communication between cells [38, 40]. 2D culture confines cells to a planar environment and restricts the more complex morphologies which showed in nature tissue. Some research group have shown cell types, including neurons, have altered metabolism and gene expression patterns in 2D cultures compared to 3D cultures, NSCs cultured on 3D systems showed the more natural morphology and better expanding instead of spreading as in the case of 2D culture [41].

Table 1: A comparison of cell/tissue behavior under 2D and 3D culture conditions [42].

Feature/function	**In 2D**	**In 3D**
Tissue-specific architecture	Poor	Rich
Cell morphology	Flat, extended	Round, contracted
Interactions	Limited	Multiple
Cell motility	Fast, free	Slow, restricted
Cell adhesion	Weak	Strong
Cell growth	Directional	In all directions
Cell proliferation	High	Low
Apoptosis	Induced	Tissue-like
Intracellular stiffness	An order-of-magnitude higher in 3D	
Cell polarization	Partly	Full
extracellular matrix remodeling	Absent or poor	Present
Fluid perfusion	1D	3D
Signaling and diffusion	Asymmetric	Nearly symmetric
Metabolic rate	High	Low
Cell survival when exposed to cytotoxic agents	Low	High

3D cell culture system creates a growth environment that mimics the native tissue as close as possible for cell growing in vitro since cells commit to their fate by deriving information from this environment. 3D model can provide not only spatial space and more surfaces for cell attachment, but also uniform cell suspension. Diffusion of nutrients, bioactive factors, and oxygen through 3D materials need to be sufficient for the survival of large numbers of cells in the expanding time [43]. Thus, creating 3D cell culture system to get desirable NSCs for cell therapy is a

better solution for cell therapy. Based on the material science and cell biology, an optimal environment for cultured cells can be created and tailored.

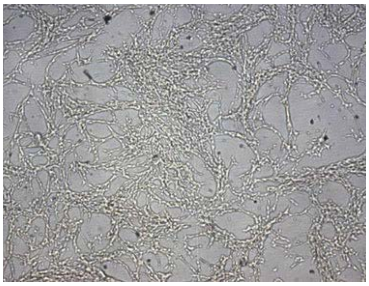


Figure 4: Adherent cultures derived from adult SVZ cells
[31] .Cells attached to the bottom of the vessel and formed a monolayer of cells 70% confluent

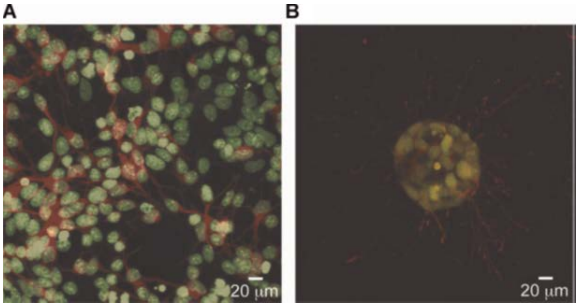


Figure 5: Cell cultured on 2D and 3D
Neural cells form monolayer in 2D (A) and neurospheres formed in 3D (B) when the cells from (A) cultured in hydrogel and extend processes in three dimensions. Green indicates cell bodies and red for b-tubulin in axonal extensions [38].

1.2.2 3D Cell Culture Mimic the Niche of NSC

The micro-environment in where stem cells reside are denoted as niche, which is specialized for each type of stem cells and composed by stem cells, supportive cells and ECM. The Niche can either provide extracellular cues to allow stem cell survival and identity, or regulates stem cell behavior, or maintain a balance between quiescence, self-renew and differentiation [44, 45]. The components of the niche can influence the cell behavior, moreover, the interactions between stem cells and theirs niches are also reciprocal since the stem cells can receive the signals from niche, remodel the niche and secrete the ECM components [46].

1.2.2.1 Neural Stem Cell Niche

The neural stem cell niche is a complex and dynamic microenvironment, which includes the brain ECM, highly concentrated blood vessels, microglia, and more differentiated neural cells [47]. Extracellular factors such as growth factors, neurotransmitters, morphogens, chemokines and the ECM are critical components of the niche [48]. Even the exact detail of the cells contacts and micro-environment are still poorly defined, it is proposed that the niche of NSCs is a constantly changing micro-environment played by many factors and cell-cell interactions. The close contact with both endothelial cells (ECs) and astrocytes are important to NSC self-renewal and differentiation [49-51]. Mature neurons also influence the fate of new NSCs through a feedback loop [52]. Soluble extracellular factors (growth factors, nertotrophic factors or cytokines) are constantly synthesized,

secreted, transported, and depleted. Some of them may temporarily bind to components of the ECM to further modify the niche. The interactions of these factors make the dynamic processes of niche that create an environment in which NSCs must constantly sense and respond in real time. Among the multiple factors, the elasticity or compliance of the matrix substrate, and the biochemical composition and dimensionality of the matrix play an essential role to regulate NSCs.

1.2.2.2 ECM in Niche

ECM is a dynamic and complex network of macromolecules with specific physical and biochemical property [53]. It acts as compartment of cells and support the organ development, function and repairing by modulating the production, degradation and remodeling of its components. Various components of the ECM confer well-defined physical, biochemical and biomechanical properties to ECM [54] of niches and they are the key players of niche. Physical properties of ECM can influence anchorage-related biological functions, like cell division, cell differentiation and cell migration [54]. The biochemical properties of the ECM provide the signaling pathways by either direct or indirect manner [55]. Based on the knowledge of the molecular composition of ECM, the 3D cell culture system can be finely tuned for providing the most appropriate artificial niche for NSCs in vitro.

1.2.2.3 ECM Of CNS

The brain ECM, in where the NSCs exist, has its specific components and property. First, the ECM in the brain is quite soft comparing with other tissues. The stiffness of brain ECM is within the range of 0.1-1kPa [56], where-else in muscle is 8–17kPa [57]. The density of the matrix affects the cell's ability to migrate, and the stiffness of the matrix has been shown to impact the differentiation of MSCs (Figure 6) [58, 59]. Second, the composition of ECM molecules in CNS is different from other tissues. Different types of proteoglycans are abundantly expressed in the adult brain and localized to intercellular spaces between neurons and glia. Proteoglycans of the lectican/hyalectan-family and their binding partners, hyaluronan, link proteins and tenascins are the main composition of ECM of CNS [60]. The ECM of CNS shows the predominance of hyaluronan and chondroitin sulfate proteoglycans (CSPG) [61, 62] instead of otherwise frequent ECM molecules, like fibronectin or collagens [63, 64] in ECM of other tissues. Further,

The ECM is constantly being remodeled by NSC neighbors (e.g. glial cells and Ecs) through matrix synthesis and degradation [65-67].

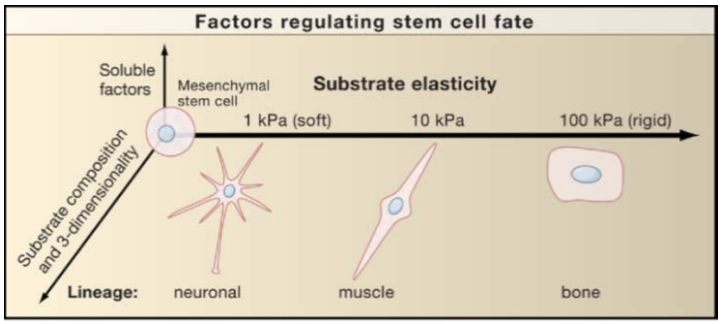


Figure 6: Matrix substrate influence the differentiation of stem cells
The differentiation of human MSC showed that soft matrices favored differentiation of MSC into neuronal-like cells, moderate elasticity promoted myogenic differentiation, and a rigid matrix stimulated osteogenic differentiation [59, 68]

1.3 3D NSCs Hydrogel Cell Culture Systems

The 3D hydrogel can provide a nature-like microenvironment to regulate cell fate through either cell–cell or cell–matrix interactions due to their tissue-like flexibility, interstitial flow and diffusive transport characteristics similar to native tissues.

1.3.1 Hydrogel Mimics the ECM of CNS

Hydrogels are cross-linked macromolecular networks formed by hydrophilic polymers swollen in water or biological fluids. Hydrogels are non-cytotoxic, it allows high oxygen and nutrient permeability, facile migration of cells and diffusion of biomolecules while maintaining a physical structure [38, 69, 70] . Many kinds of hydrogels can be formed under mild, cytocompatible conditions, with easy modification to possess cell adhesion ligands, desired viscoelasticity, and degradability [71]. The physico-chemical (electrical charge and pore size) [72-74] and mechanical properties (stiffness, tensile strength) [75, 76] can be adjusted by changing the type and ratio of polymers, the concentrations of polymers and the crosslinking density [76]. Adding different types of supporting peptides or trophic factors and adjusting their concentration can make hydrogel more optimal for cell growth. By combing all these technologies, series of hydrogels used for cell culture can be formed from a vast array of natural and synthetic materials, with a broad spectrum of mechanical and chemical properties [77]. Another important attraction of hydrogels being widely utilized as 3D cellular microenvironments is their ECM-like biophysical property [78-80]. Many hydrogel have similar mechanical properties as ECM of CNS. Thus, hydrogels are a highly attractive material for developing artificial ECM of NSCs [59].

1.3.2 Hydrogel Formation

Hydrogels with 3D network structures obtained from synthetic and/or natural polymers, can absorb and retain significant amount of water [81]. It is formed by the hydrophilic groups or domains present in a polymeric network upon the hydration in an aqueous environment. Hydrogel is prepared by dissolving hydrophilic linear polymer in water due to the thermodynamic compatibility of the polymer chains and water. Such soluble branched polymer is called sol. In the presence of crosslinking points or conditions, linking of macromolecular chains together results in increasing the size of the branched polymer with decreasing solubility and infinite polymer in turn formed, which is called gel or network. The transition from the sol to the gel is called gelation [82, 83]. Gelation can occur by physical and/or chemical crosslinking. In 3D cell culture process, seeding the cells is done by mixing the cell suspension with sol followed by the gelation. Every step of this process should be compatible with cell survival and should induce a minimal stress to cells. Therefore, the gels that gelation occurs under mild conditions will the preferable for cell culture.

Chemically cross-linked hydrogels is formed by the linear polymer chains covalently bonded with each other via crosslinking agents. Normally this leads to a permanent hydrogel, that means the polymer is no longer soluble in solvents and heating melt-process can only degrade the polymer. Chemically cross-linked hydrogel result in a network which can`t be reshaped or resized, the cross-linker used may be toxic. Physical gels can be strong gels and weak gels depending on the physical bonds between polymer chains. Some physical gels can be reversible when the networks are held together by molecular entanglements, and/or secondary forces including ionic, hydrogen bonding or hydrophobic interactions [83-85]. The hydrogels, whose gelation can be induced by either physical crosslinking such as in the presence of ions and the temperature change, or chemical crosslinking such as irradiated by light, will be chosen to design the cell culture system [86]. Changing temperature leads to the polymer molecules rearranging from random coil to helix, then the helices assemble in clusters and join together by the untwined regions. In presence of ions, the sections of the polymer backbone which carry the charge bind with the ions of opposite charge. Photo-cross-linking involved in the photoinitiator molecules in solution forms radicals when irradiated by UV and cross-link the polymer chains. Among the different gels, physically cross-linked thermo sensitive hydrogels gained

considerable attention since it may undergo sol-gel phase transitions at a critical solution temperature and can be reversible with temperature change. The ion sensitive hydrogels are very attractive for the cell culture since most culture media contain certain amount of ions, which can be used as inducer of gelation instead of adding additional ions.

1.3.3 Hydrogel Control NSCs Behaviours

Many different types of hydrogel have been evaluated in cell culture or tissue engineering. The physical and biochemical properties of these hydrogels, affect cell behaviors, such as viability, attachment, proliferation and migration.

1.3.3.1 Cell viability

Cell viability is the first consideration to design a hydrogel model for cell culture. The material needs to support or improve cell viability when cell are embedded in culture substrate. The material to fabricate hydrogel should be biocompatible for the embedded cells, and the concentration of hydrogel need to be suitable for cell survival and growth as hydrogels with certain concentration can be toxic for cells. Different hydrogels including pluronic F127 (PF127), matrigel and puramatrix for human NSCs (hNSCs) have been tested. It was found that PF127, in a 30% gelated form, was toxic to hNSCs and showed less viability of hNSCs than that in 10% hydrogels. Matrigel in a 1–50% form prevented hNSCs' normal capacity for neuronal differentiation. PuraMatrix showed low toxicity when it 0.25% and retained several crucial properties of hNSCs, including migration and neuronal differentiation [87].To improve or enhance cell viability, bioactive factors like NT-3 [88], adhesive peptides like collagen and laminin-derived cell adhesive peptide [89] have been used.

1.3.3.2 Cell attachment

Cell attachment or adhesion is another concern in cell culture for further cell growing and proliferation. Cell adhesion molecules (CAMs) located on the cell surface can bind to the ECM and play the essential role between cells and their surroundings to control NSC development, including their proliferation, differentiation and migration, through extrinsic signals from the stem cell niche [90]. Cell attachment can be influenced by chemistry of hydrogel, such as hydrophobic interface or hydrophilic interface, which can change the ability of protein adsorption [91]. Chemical modifications on non-adhesive hydrogels can improve cell adhesion and modulate cellular responses. The commonly used is conjugation

of short peptide sequences to non-adhesive gels, such as RGD [92] and IKVAV [93] as well as CDPGYIGSR and CQAASIKVAV [94]. The modification can work with both synthetic and nature-derived hydrogel and improve neuronal adhesion [95, 96].

1.3.3.3 Cell proliferation

To get a big amount of NSCs for cell therapy, proliferation must be precisely controlled and promoted. Hydrogel stiffness often affects NSCs proliferation. It has been found that the proliferation of NSCs decreased with the increasing of stiffness of alginate hydrogel [97]. NSCs seeded in different gel favour different stiffness, e.g. NSCs on chitosan substrates that possessed Young's elastic moduli of 3.5 kPa exhibited the maximum proliferation [98]. Degradation of hydrogel further promoted NSCs proliferation because of the efficient nutrient and gas exchange [99]. Hydrogel modified by some peptides also influence cell proliferation [100].

1.3.3.4 Cell Migration

Cell migration is one parameter to assess the NSCs culture. During culture the NSCs can self-renew and proliferate in the gel. Individual cells will aggregate together if the new proliferated cells can`t freely move to other place, which will leads to uneven neurosphere formation depending on the cell density and proliferate rate. Uniformed spheres can result in the death of part of cells which are in the middle of big spheres. Free movement of cells allow proliferated cells distributing more evenly in the spatial space in gel, and give cells more space to develop their phonotype . Migration of cells can be influence by the gel stiffness and pore size, growth factor and ligand incorporation [101].

1.3.4 Hydrogel Design Criteria

The design of 3D cell culture system is a crucial step. Developing 3D cell culture requires the control of physical and chemical properties which may vary a lot. The gel mimicking ECM should take account of the considerations of mechanical properties, adhesive ligand, growth factors and transport kinetics as well as to be cytocompatible, reliable, and cost effective ([38, 102]. It needs to allow the exchange of oxygen and nutrients as well as growth factors for supporting cell growth. It should promote neural cell survival and proliferation while maintaining the intended function. In addition, it also needs to be suitable for large scale production and reproducible as well as can be modified for cellular recognition

motif for interaction. The gel degradation products should not have a deleterious effect on encapsulated cells [38]. Thus the designed hydrogels with all these considerations may provide artificial 3D stem cell niches in vitro to enhance NSCs growth.

1.3.4.1 Hydrogel stiffness

The hydrogel can control the cells through the mechanical strength (stiffness) of the substrate. Mechanical strength can regulate cell behaviors (Figure 7) and fate the cell development [59, 103]. Though the mechanism of mechano-transduction events is still not totally clear, but mechanical properties of hydrogels fate the stem cells has been demonstrated [59]. NSCs encapsulated within 3D alginate hydrogels demonstrated that the rate of proliferation of NSCs decreased with increase in the modulus of the hydrogels (Figure 8) [97]. The NSCs showed the greatest expression of the neuronal marker β-tubulin III within the softest hydrogels, which had an elastic modulus comparable to that of brain tissues [97] .

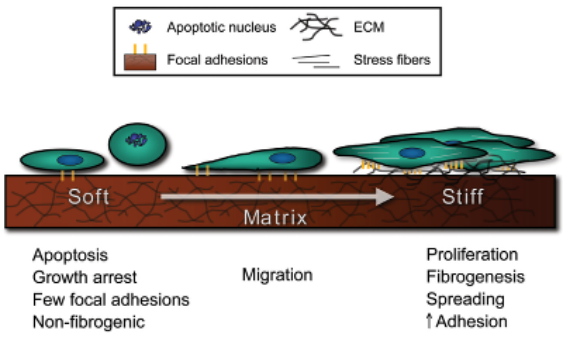


Figure 7: Effects of matrix mechanics on cell behavior
Schematic of the general changes in cell behavior observed as matrix stiffness increases. But optimal matrix stiffness varies among different cell types [104].

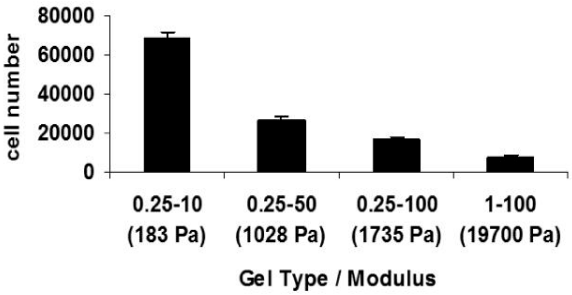


Figure 8: The influence of stiffness on NSCs proliferation
The proliferation rate of NSCs decreased with increase in the modulus of the hydrogels [97].

The result revealed that the mechanical properties of hydrogel can perform a defining influence on the proliferation of the embedded NSCs. It suggests that hydrogel mimicking the physiological stiffness of ECM may benefit cells to survive. Since the conditions of nature niche is so complicated, physical properties of hydrogel needs to be finely tuned by changing the concentration and ratio of polymers as well as the cross-linker to get the optimal stiffness for desirable proliferation rate and cell fate.

1.3.4.2 Hydrogel Microstructure and Porosity

The porosity and pore architecture of hydrogel is a key role to fabricate functional hydrogel. Embedded cells need oxygen and nutrients supply, meantime the produced waste need to be transferred to the culture media. Thus the proper porosity to allow supporting factors and nutrients as well as metabolites diffusion through hydrogel is important for cell survival and proliferation [105]. For this goal, the pore size needs to be adjusted according to cell density and cell types. For example, the mass transfer rate of nutrients should be higher when the cell density is higher. Gradient also need to be considered since the cells seeded far away from the border of the matrix may have low transfer rate or need long time to get nutrients. This suggests that hydrogel with high surface area to volume ratio may perform better in providing the right conditions to support viable cells. Further, pore size is also very important for cell migration. Proliferated cells from each individual cells will retained together if the pore size is too small for them to move, this will lead to neurosphere forming in cell culture which lead to the uniform cells distribution especially for direct transplantation used hydrogel. Despite of these, the degree of porosity may also have an effect on the mechanical properties, e.g. the stiffness decrease as porosity increases. Different cells favour different optimal pore size. The movement of MSC in 3D synthetic scaffold was observed for an intermediate pore diameter rather than the largest pore diameter [106]. Degradable hydrogel has revealed that the highly porosity significant increase in the expansion rate of NPCs cultured in degrading alginate hydrogels versus NPCs cultured in standard, i.e. non-degrading [99]. In addition, increased porosity can have a beneficial effect on the diffusion of nutrients and oxygen, especially in the absence of a functional vascular system. Pore size can be modified by using many techniques which include solvent casting/particle leaching, freeze-drying, gas foaming, and electro-spinning [107].

1.4 Hydrogels for NSCs Cell Culture Systems

Hydrogels can be classified into synthetic hydrogel or natural hydrogel based on their derivation, or non-degradable and biodegradable hydrogel depending on the possibility of degradation. They also can be divided into physical crosslinking hydrogel or chemical crosslinking hydrogel according to the gelation mechanisms. For intending to CNS repairing or NSCs cell culture, many biomaterials have been investigated (Table 2).

1.4.1 Synthetic Hydrogels

Many different synthetic hydrogels have been tested for CNS repair. Biodegradable hydrogel includes poly(lactic acid) (PLA) [108], poly(lactic-co-glycolic acid) (PLGA) [109], poly(ethylene glycol) (PEG) [110]. The non-biodegradable including the poly(2- hydroxyethyl methacrylate) (pHEMA) [111] and poly(hydroxypropyl methacrylate) (pHPMA) [112]. The advantage of synthetic hydrogel is that they can be easily tailored, possess controllable degradation and microstructure and high mechanical properties. But lack of biological moieties and chemical used in the process of fabrication which can lead to the unexpected reaction with the cells or tissue, needs to be considered for the clinical use.

1.4.2 Natural Derived Hydrogel

Hydrogels derived from natural materials have a similar structure as the ECM of human tissues. Natural hydrogels are made of polymers similar to the biological macromolecules and can perform specific functions in a demanding environment. Many of them can be processed under mild conditions compatible with cell survival [113] and provide a milieu of endogenous signals that promote the cellular interactions, e.g. matrigel [114] and collagen [115] have been widely used in three-dimensional tissue culture and regenerative medicine. Commonly used natural hydrogel also includes hyaluronic acid (HA) [116], chondroitin sulfate [117], fibrin [118], fibronectin [119], alginate [120], agarose [121], chitosan [122] and silk [123]. Gellan gum [124] also got more attention recently. The risk of Natural hydrogels for regenerative medicine may arise from undefined compositions, batch-to-batch variation, and risk of immune rejection for animal-derived materials.

Table 2: hydrogel [125]

Material	Source	Inherently cell adhesive	Injectable	Gelation mechanism	Comments
Matrigel™	Mouse tumor cells	Yes	Yes	pH and temperature changes	Primarily used as a cell carrier but unlikely to be approved for human use
Collagen	Animals	Yes	Sometimes	Chemical crosslinking, pH and temperature changes	Highly biocompatible and used to mimic the natural ECM
Hyaluronic acid	Animals, bacteria	No	Yes	Electrostatic interaction	Often chemically derivatized to form injectable gels and has mostly been used for brain injuries
Fibrin	Blood	No	Yes	Enzymatic crosslinking	Extensively tested in vivo and shown to be suitable for cell and biomolecule delivery

Material	Source	Inherently cell adhesive	Injectable	Gelation mechanism	Comments
Alginate	Algae	No	Sometimes	Ionic crosslinking	Used as a preformed scaffold for cell transplantation and regeneration studies
Agarose	Seaweed	No	No	Temperature change	Mostly used as a preformed scaffold and has shown promise in promoting regeneration
Chitosan	Insects and crustaceans	Sometimes	Sometimes	Ionic or chemical crosslinking	Material is widely available in nature and has been extensively derivatized to form both crosslinked and injectable gels
Dextran	Bacteria	No	Sometimes	Chemical crosslinking	Recently developed into macroporous scaffolds
PEG	Synthetic	No	Sometimes	Chemical and photo-crosslinking	Used for controlled studies of scaffold degradation and cell adhesivity
PHEMA	Synthetic	No	No	Chemical crosslinking	Supports biomolecule delivery and has highly tunable mechanical properties
PHPMA	Synthetic	No	No	Chemical crosslinking	Can be functionalized with peptide domains and has similar mechanical properties to CNS tissue

Optimal hydrogel can be fabricated by either single polymers or hybrid polymers and combined with different growth factor, tropic factor, chemical molecules, or peptide to enhance cell attachment, growth, proliferation or differentiation. In this paper, we focus on the gellan gum and konjac gum based hydrogels.

1.4.2.1 Gellan Gum (GG)

Gellan gum is composed of tetrasaccharide (1,3-b-D-glucose, 1,4-b-Dglucuronic acid, 1,4-b-D-glucose, 1,4-a-L-rhamnose) repeating units and contain one carboxyl side group [126, 127]. Gellan gum is a linear anionic polysaccharide derived from microbial fermentation product of sphingomonas elodea and two isoforms exist, one acylated form that produces soft hydrogels and another deacylated form that produces hard and brittle gels [128]. The difference of their physical properties of both forms can be seen inTable 3. Gellan gum has been used as food gelling or thickening agent for decades.

Table 3: Difference of two isoforms of gellan gum

	Low Acyl	**High Acyl**
Gel characteristics	firm, non-elastic, brittle gels	soft, elastic, non-brittle gels
Molecular weight	2 - 3x10^5 Daltons	1 - 2x10^6 Daltons
Solubility	Cold or hot water	Hot water
Set Temperature	30º - 50ºC (86º - 122ºF)	70º - 80ºC (158º - 176ºF)
Thermoreversibility	Heat stable	Thermo-reversible
Hydration temperature	Sensitive to ions particularly to divalent ions. inhibited by soluble solids and low pH	Relatively insensitive to ions. inhibited by soluble solids and low pH

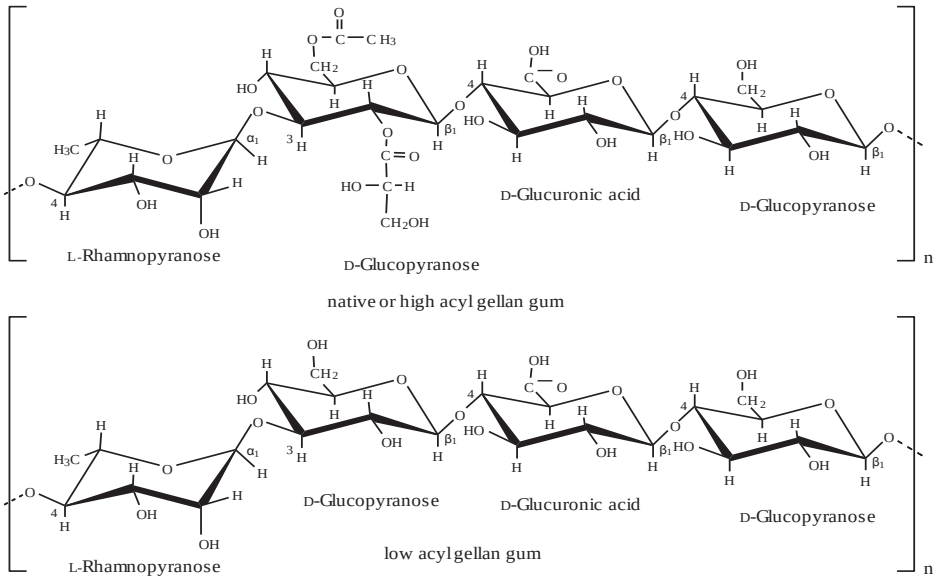


Figure 9: Structure of isoforms of gellan gum [129]

Hydration of gellan gum can be done by dissolving the powder in water when heated. Hydration of both forms can be influenced by soluble solids and low PH. Hydration of low acyl form is very sensitive to cations whereas not in case of high acyl forms [130].

Both native and deacylated GG are capable of physical gelation. Gelation occurs when temperature change or in the presence of cations. GG in sol are in random-coil conformation at high temperature and the transition to double-helix occurs upon cooling. Afterwards, the double-helices self-assemble in clusters of anti-parallel structures and junction zones are formed. Following this process, untwined regions of polysaccharide chains link the junction zones together in turn form a 3D network to create gel [131], which is thermo-reversible [128]. The gel can be melted at 90-100°C. Gel can be reformed instantly when its setting point has been reached. Sol-gel transition started to occur at approximately 36ºC [132] and the setting temperature can vary from 35-50°C influenced by the concentration of gellan gum, type and concentration of cations and the presence of other dissolved solids. Partial aggregation may form an ordered structure on low concentrations, but there is no real gel formation because the number of helical aggregates does not give rise to a continuous network in the whole volume [133]. The stable hydrogel can be obtained by adding cations, which can shield the electrostatic repulsion of the negative charged carboxyl group and thereby help the tight binding and aggregation of helices. Especially the divalent cations can bind together two carboxyl groups producing stronger gel [133-135]. Some experiments also suggested that cations affect gelation differently, K+ was more than Na+,

and Ca2+ more than Mg2+ [130, 132, 133] (see Figure 10). Gel strength increased while the concentration of polymers and cations increase (see Figure 11), except Ca^{2+}. At a calcium concentration above 0.1%, gel strengths begin to decline and the gel is no longer thermo-reversible (Figure 10).

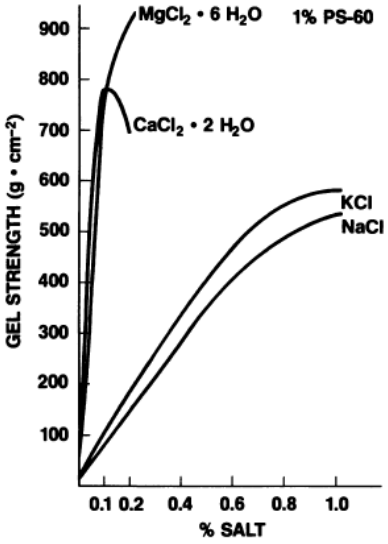


Figure 10: Effect of cations on gellan gum gelation [130]

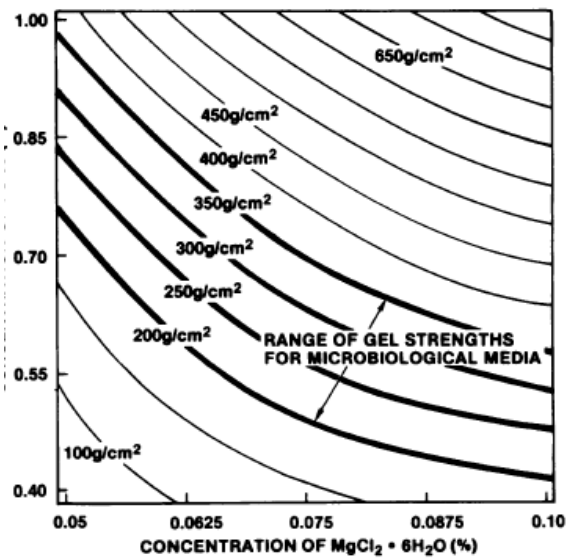


Figure 11: Gel strength as function of GG and Mg^{2+} concentration [130]

GG gel also can be obtained via chemical crosslinking. A novel gellan chemical hydrogel with tunable physicochemical properties has been obtained by crosslinking the polymer chains with L-lysine ethyl ester moieties [136]. Gel formed by adding 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC) and N-hydroxysuccinimide (NHS) to the hot solution of gellan (T $\approx$ 50 ° C) and in turn the crosslinking occurs between the carboxyl groups of gellan polymer chains and the amine groups of Lys. In this way, the disordered structure of the polymeric chains is maintained by the chemical linkages also when the temperature is lowered to room temperature. Physical gel and chemical gel have difference not only in the mechanism of gelation, but also in the structure (see Figure 12) and storage modulus [136].

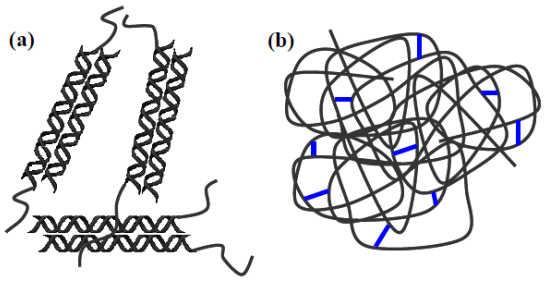


Figure 12: GG Schematic representation
(a)physical gel of Gellan, (b)chemical gels of Gellan [136]

The physical gelation properties make gellan very attractive as biomaterial. Comparing to other hydrogels, GG showed many advantages for its intended use, e.g. high gel strength and clarity, low use level forming gel, excellent stability, easy production, thermo-reversible gel. Despite of these, the gel texture can be easily modified by adjusting the ions or polymers concentration. Chemical modifications can be done on carboxyl group easily to improve its mechanical properties and stability in vivo [137]. The sol–gel transition required temperature change and presence of cations are compatible with a physiological environment and with cell entrapment. For cell culture systems, most media already contain certain amount of cations, like Na+, K+ or Ca2+. Without any additional cations, many GG solutions can form the gel immediately when it is mixed with cell suspension under room temperature and gel maintained under 37ºC during cell culture. GG also show the thermal stability to withstand several autoclaving cycles. Most enzymes had no effect on viscosity or gel strength [138]. Further, shear-thinning property can provide a remarkable suspending possibility [139] and make GG possible to gelate in situ which is very interesting for injectable hydrogel. Degradation of GG can be done by the enzyme galactomannanase [140]. The rate of degradation is influenced by the number of acyl groups, low acyl GG gel degrade more slowly than the high acyl forms [141].

GG has been used as stabilizer and gelation agent in food and personal care as well as in pharmaceutical industry to modify or control the rate of release of active materials of drug. In biotechnology area, GG can be used as growth media for bacteria or medium plant tissue cultivation [142]. Furthermore, GG has been recently proposed as cells supports in tissue engineering. Encapsulated human nasal chondrocytes were cultured in GG based hydrogel, which serve as a cell support in the field of cartilage regeneration [132]. Modified GG hydrogels with tunable physical and mechanical properties support the growth of fibroblast [143]. Gellan gum has been used to develop a novel hybrid tissue engineering-based scaffold for spinal cord injury repair by supporting the in vitro culture of oligodendrocyte like cells [144]. Modified GG (GG-GRGDS) had a profound effect on NSPC morphology and proliferation, distinct from that of NSPCs in GG alone [145].

1.4.2.2 Konjac Glucomannan (KGM)

KGM has high-molecular weight and is a water-soluble, non-ionic and natural polysaccharide [146]. It is a linear random copolymer of glucose and mannose and derived from the tubers of amorphophallus konjac [147, 148]. D-glucose and D-mannose is in a molar ratio of 1.0:1.6 and linked by ß-1->4 glycosidic bonds [149]. It consists of lower acetyl group (approximately 1 acetyl group per 17 residues) at the C-6 position and certain short-chain branches at the c-3 position of the mannose [148, 150, 151]. KGM is regarded as non-digestible polysaccharide due to ß-glycosidic linkages between the glucose and mannose building blocks (ß-1->4 linkages in the main chain and ß-1->3 linkages at the branch points). Therefore, it has been used as weight reducing food. High molecular weight with ranging from 200,000 to 2,000,000 Da, can vary with origin, method of processing, and storage time.

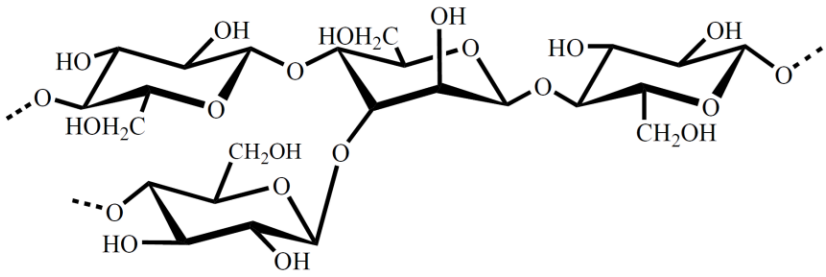


Figure 13: The chemical structure of konjac glucomannan [152]

High water solubility conferred by attached acetyl groups makes KGM as a soluble fiber and the solution is a pseudo-plastic liquid, characterized by shear thinning and high viscosity [148]. Viscosity of konjac solution can be influenced by its concentration but not by pH (pH 2-8) and salt concentration (0-10% w/v NaCl). The konjac solution maintained its viscosity under sterilization process (121°C at 15lb/in^2) for 30 min, whereas the viscosity decreased in the same condition with the presence of 2.5% NaCl [153].

KGM particle can swell to about 200 times as its original volume. This swelling is caused by the extremely long thread-like macromolecules which tangle together, when water molecules enter and are absorbed into the chain of KGM. High molecular weight, high density and extreme swelling properties result in the highly viscous solutions. It has been reported that the its viscosity is greater than that of guar gum, one of the most viscous of the dietary fibers [154] .

Gelation of KGM occurs by forming a network structure supported by hydrogen bonding. The solution of KGM is never gelatinized with any other agents than alkalis. Gel forms in the presence of mild alkali and enhanced by heating [155]. Heat stable and thermally reversible elastic gel [148] can be formed with addition of a mild alkali such as calcium hydroxide. Since the acetyl groups inhibited the KGM to form gel, mild alkali present in KGM solutions can cleave the acetyl groups and produce deacetylated KGM, which is able to build junction zones through hydrogen bonds [156]. Although the details of gelation mechanisms are not yet fully understood, the research have shown that the rate of gel formation is dependent on concentration and molecular weight of KGM, temperature, degree of acetylation, alkaline concentration and pH [157-159]. Rheometric studies revealed that KGM with different molecular weights exhibited different gelation characteristics. Lower molecular weights showed a decrease in both the storage shear modulus (G') and loss shear modulus (G") during gelation at temperatures above 75 º C, whereas not in that case of native KGM [158]. At a fixed alkaline concentration, increasing concentration of KGM or temperature can accelerate the gelation time, but increasing degree of acetylation delays it. This indicates that the presence of acetyl groups exerts a strong influence on gelation behavior of KGM [160]. Acetylated KGM needs longer time than that of native sample to deacetylate and gelatinize. Acetylated KGM forms finally more elastic gels due to that junction zones are more homogeneously distributed and more numerous, which leads to more elastic gels in slower gelation processes [161]. A pH range necessary for gelation is 9 to 10 [162]. The KGM gel can be solubilized under mild conditions with reagents (urea) which can break the hydrogen bond. This can indicate that the gel dos not consist of any chemical cross-links [150].

KGM gel has the capacity to modify the rheology of aqueous media to which they are added, even at low concentrations, which is important for their stabilizing, thickening and gelling propose [160]. Secondly, due to their biocompatibility, solubility, potential for modification, and innate bioactivity, KGM also involves in many other applications in drug delivery systems such as encapsulation or controlled release of drugs [163] e.g. KGM/xanthan gum mixtures are used as excipients for controlled drug delivery systems [164].

1.4.2.3 Gellan and Konjac Hybrid Gum (GG/KGM)

The interactions of KGM with other hydrocolloids have been carried out. The KGM mixed with xanthan, and κ-carrageenan has been studied. Synergistic interaction of KGM with xanthan gum occurs and forms thermo-reversible gels when KGM is mixed with xanthan gum in an optimal ratio of 1:1 in the absence of electrolyte. This ratio changed to 1:2 KGM/xanthan in the presence of 0.04 mol/dm3 NaCl, and the interaction of mixed polymer was described by Williams, P. A.,&Phillips [165-167]. Mixed gel using X-ray diffraction and X-ray fibre pattern indicate the evidence of intermolecular binding, but the research of more details of the gelation mechanisms is still ongoing [168]. The interaction between KGM and κ-carrageenan was tested [169]. Since both GG and carrageenan belong to helix-forming polysaccharide, it has been thought that the synergistic interaction may occur between GG and KGM. It was found that maximum storage modulus appeared at a mixing ratio of GG/KGM=0.6:1.0 (below 15ºC with total polysaccharide concentration 1.6%) and GG/KGM =0.3/0.5 (at 0ºC with total polysaccharide concentration 0.8%).The result indicated that the synergism of GG and KGM occurred at much lower temperatures than the coil-helix transition temperature, where individual helices in GG molecules sufficiently aggregated. The gel formed by KGM chains attaching the surface of the self-aggregates core of GG helices, rather than individual GG helices [170]. Another investigation was done by rheology and texture analysis on mixture of GG and KGM. The result revealed that mixed sols is a non-newtonian fluid and shear-thinning. The mixture showed the fluid characteristics at KGM/GG ratio bigger than 5:5, the viscosity of mixed sols increased quickly with the increasing ratio of KGM. The storage modulus of mixed gel increased with the increasing concentration of GG, which indicates that GG play a main role in gel stiffness. The gel texture can be influenced by cations. In a certain concentration range, the increase of cations might promote the gel's hardness and cohesiveness, maximum hardness is achieved when the monovalent cations were added at the concentration of 150 mM, while the bivalent cations are at the concentration of 10mM [171].

In my study, we try to make the GG gel more permissive to enhance cells growth. The hypothesis is to get a more permissive by mixing the KGM and GG through the synergistic interaction with low concentration of GG, or by using the long fiber-KGM to make the pore size bigger in the mixed gel.

2 Aim of the Study

The aim of this study is to develop a three-dimensional (3D) cell culture system which can mimic the natural extracellular matrix (ECM) of CNS in vitro to support the growth and fast proliferation of neural stem cells.

As we know that three dimensional of the natural tissue have characteristic biophysical and biomechanical signals for the embedded cells. The biophysical signals in 3D extracellular matrix (ECM) can influence cell functions like migration, adhesion, proliferation and gene expression. The ECM functions as a biological adhesive substrate that maintains the normal cytoarchitecture of different tissues and defines the key spatial relationships among dissimilar cell types. Since the hydrogel has similar properties as ECM of CNS, the hydrogel was used as substrate to create the 3D cell culture system for supporting NSCs proliferation. By designing the self-assembling hydrogel with shear-thining property, by finely adjusting the biochemical and biophysical properties through changing the gel types, ratios and concentrations, by adding different types and adjusting concentration of trophic factors, to get the optimal conditions of 3D hydrogel systems. Under this optimal conditions cell culture will be done to identify and improve the 3D hydrogel model which can support the growth of neural stem cells to get the desirable cell phenotypes or cell number

3 Materials and Methods

3.1 Gel Solution

3.1.1 Gellan Gum (GG) Solution

Gellan gum solution is made from Phytagel which is a trademark of Sigma-Aldrich Biotechnology LP and Sigma-Aldrich Co ARO. The solutions with different concentration of GG in w/v (0.5% 0.8% 1.1% 1.4% 1.6%) were made by dissolving the phytagel powder in deionized water with conductivity less than 18.2 MΩ·cm from Millipore. After autoclaving (121ºC, 30 minutes) the gel solutions were cooled down at room temperature for 24 hours and afterwards the physical property were tested and the cell cultures were done by using the gels as 3D substrate.

3.1.2 Hybrid Gel Solution of Gellan Gum and Glucomannan (GG/KGM)

The KGM product used here is from the company Now. Gel solutions were made by mixing the phytagel and KGM powder in different ratio with different concentrations. Series of GG/KGM solution were prepared with GG/KGM of % w/v in 0.6/1.0, 0.8/0.8, 0/1.0, 1.1/0.3, 1.1/0.5, 1.1/0,7, 1.1/0.9, 1.4/0.2, 1.4/ 0.4, 1.4/0.6 and 1.4/0.8. Gel solutions were autoclaved (121ºC, 30 minutes) and cooled down at room temperature for 24 hours, the physical property were tested and later cell cultures were done by using the gels as 3D substrate.

3.2 Rheology of Gels

The study of dynamic rheology was done by using a 20mm parallel plate on a Discovery HR-3 Hybrid Rheometer. 1-2 mm gel was prepared and load on the stage of the rheometer. Both oscillation amplitude sweep (OAS) and oscillation frequency sweep (OFS) were employed. The soaking time were set up at 2 minutes. The OAS was set up by holding the temperature at 37 °C and constant frequency (1 Hz) while increasing the stress level from 1 to 10 Pa. The linear viscoelastic region (LVR) profiles determined by the OAS were then applied in OFS sweep. OFS were performed at 50% of their LVR stress levels and temperature (37 °C), the oscillatory frequency was increased from 0.1 to 100 Hz. Storage modulus G’ and lost modulus G’’ were recorded. Gels without media and gels with 100 ml media which mimic the culture conditions were tested.

3.3 Cell Culture

3.3.1 Neural Stem Cells Culture

Except the 2D control wells, 24 wells plates were coated first by 1.4% GG solution 130µl per well to ensure that the cells grow in 3D model. 2D control wells were coated by 1% martrigel (Millipore). NSCs proliferation medium were prepared by mixing 500ml ReNcell NSC maintenance media (EMD Millipore) with 5ml Gibco Antibiotic-Antimycotic (100X). Basic fibroblast growth factor (bFGF) and epidermal growth factor (EGF) from Sigma-Aldrich were added to the final concentration of 20ng/ml (for resuspension, cell seeding and 2D controls) and 30ng/ml (for 3D cell culture on gel). Millipore NSCs of sub passage P7 were detached by StemPro® Accutase from Life Technologies. After cell counting and centrifugation, NSCs were resuspended in medium at the cell density of $2.5*10^6$ cells per ml. Cell seeding was done by mixing 100µl gel solution (GG of 0.8%, 1.1%, 1.4%, and hybrid GG/KGM with different composition and concentration GG/KGM by % w/v of 1.1/0.3, 1.1/0.5, 1.1/0.7, 1.1/0.9 1.4/0.2, 1.4/0.4, 1.4/0.6, 1.4/0.8) with 100µl cell suspension following mild vortex and 200µl of mixture per well were plated into the pre-coated well. Final concentration of NSCs in 3D gels is $1.25*10^6$ cells per ml and 250K NSCs per well. 2D control was done by seeding 100µl NSCs suspension per well in 100µl media in pre-coated wells. After half hour incubation (37ºC and 5% CO_2) the gels with cells were covered by 500µl medium (bFGF and EGF 30ng/ml). The medium were changed each other day and the plates were incubated in incubator with 37ºC and 5% CO2 for 6 days. The microscope pictures were taken on day 1 and day 6. The confocal pictures were obtained after 6 days cell culture following cell fixing by 4% formaldehyde and phalloidin stain for actin (green) as well as dapi stain for nucleus (blue). Live dead stain was performed with same method but with cell density of $2.5*10^6$ cells per ml in gels in three different gels. All stain kits are purchased from Life Technologies.

3.3.2 Mesenchymal Stem Cells

Except the 2D control wells, 24 wells plates were first coated by 1.4% GG solution 130µl per well to ensure that the cells grow in 3D model. MSCs proliferation medium were prepared by mixing 500ml MSC medium with 10ml fetal bovine serum (FBS) and 5ml 100X MSC growth supplement serum free (MSCGS-sf), all these products were purchased from ScienCell. Human bone marrow derived mesenchymal stem cells (MSCs) purchased from Sciencell Research Laboratories

were used. Sub-passage of P4 was detached by trypsin/EDTA (ScienCell). After cell counting and centrifugation, MSCs were re-suspended in proliferation medium with the cell density of $2.5*10^6$ cells per ml. Cell seeding were done by mixing 100µl gel solution (GG of 0.8%, 1.1%, 1.4%, and hybrid GG/KGM with different composition and concentration GG/KGM by % w/v in 0.6/1.0, 0.8/0.8, 1.1/0.3, 1.1/0.5, 1.1/0.7, 1.1/0.9 1.4/0.2, 1.4/0.4, 1.4/0.6, 1.4/0.8 and 1% KGM) with 100µl cell suspension following mild vortex, 200µl mixture of cells and gel were plated into the pre-coated well. Final concentration of MSCs is $1.25*10^6$ cells per ml in the gels and 250K MSCs per well. 2D control was done by adding 100µl (250K cells) cell suspension and 100µl medium per well directly. After half hour incubation (37ºC and 5% CO2) all samples were covered by 500µl medium. The medium was changed each other day and the plates were incubated in incubator with 37ºC and 5% CO2 for 6 days. The microscope pictures were taken on day 2 and day 6. The confocal pictures were obtained after 6 days cell culture following cell fixing by 4% formaldehyde and phalloidin stain for actin (green) as well as dapi stain for nucleus (blue). All stain kits are purchased from Life Technologies.

3.3.3 Meningeal Fibroblasts

Meningeal Fibroblasts (MFs) was provided by the Wenlab in VCU. The culture medium contained 10% FBS and 1% antibotics were prepared by mixing 500 ml DMEM high glucose (Life Technologies) with FBS (Life Technologies) and Gibco Antibiotic-Antimycotic, to final concentration. Following cell culture, trypsin/EDTA (ScienCell) detachment, cell counting, centrifugation and re-suspension, P4 MFs seeding were done by mixing 100µl gel solution (GG of 0.8%, 1.1%, 1.4%, and hybrid GG/KGM by % w/v of 1.1/0.3, 1.1/0.5, 1.1/0.7, 1.1/0.9 1.4/0.2, 1.4/0.4, 1.4/0.6, 1.4/0.8, 0.6/1.0.0.8/0.8 and 1% KGM) with 100µl cell suspension following mild vortex, 200µl mixture (cells and gel) per well were plated into the 1.4% GG solution pre-coated well. The final concentration of MFs is $2*10^6$ cells per ml in the gels and 400K MSCs per well. 2D control was done by adding 100µl cell suspension in 100µl medium per well directly. After half hour incubation (37ºC and 5% CO2) the all samples were covered by 500 µl medium. The medium were changed each other day, and the plates were incubated in incubator with 37ºC and 5% CO2 for 6 days. The microscope pictures were taken on day 1 and day 6. The confocal pictures were obtained after 6 days cell culture following cell fixing by 4% formaldehyde and phalloidin stain for actin (green) as well as dapi stain for nucleus (blue). All stain kits are purchased from Life Technologies.

4 Results

4.1 Rheology

To get the desirable stiffness of the gel for cell culture systems is very crucial step. In this experiment, the hydrogels of pure GG and hybrid GG/KGM of different concentration and composition in presence or absence of media were tested by OAS and OFS scan. Samples with media or PBS were prepared according to the cell culture condition, for example, 100µl sol mixed with 100µl media. Then the gels were plated out on the flat glass plate and kept at room temperature for 24 hours. The stiffness was tested by the rheometer with OAS and OFS swap.

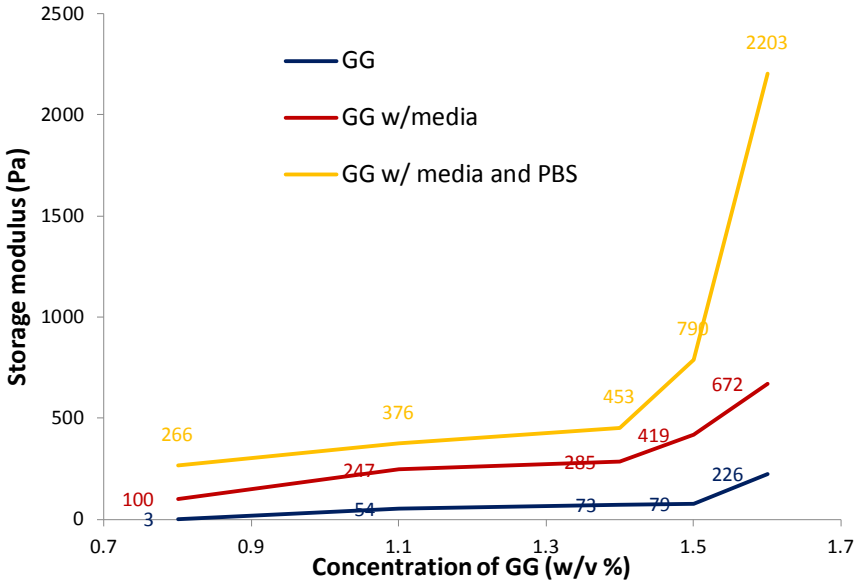


Figure 14: Effect of polymer concentration and medium/solvent on GG modulus
Blue line (pure 100 ul GG)
Red line (100ul GG mix with 100ul maintaining medium of NSCs)
Yellow line (100ul GG mix with 50ul maintaining medium of NSCs and 50ul PBS)

The storage modulus of pure GG gel increases with the increasing concentration of GG polymers (blue). In the presence of media, the storage modulus of each concentration increased comparing to corresponding pure GG in absence of the medium, and storage modulus increased with the increasing concentration of polymers (red). After mixing with PBS, the modulus increased much higher (yellow line). The result indicates that the ions in the media and PBS promote the gelation and increase the stiffness of GG gels.

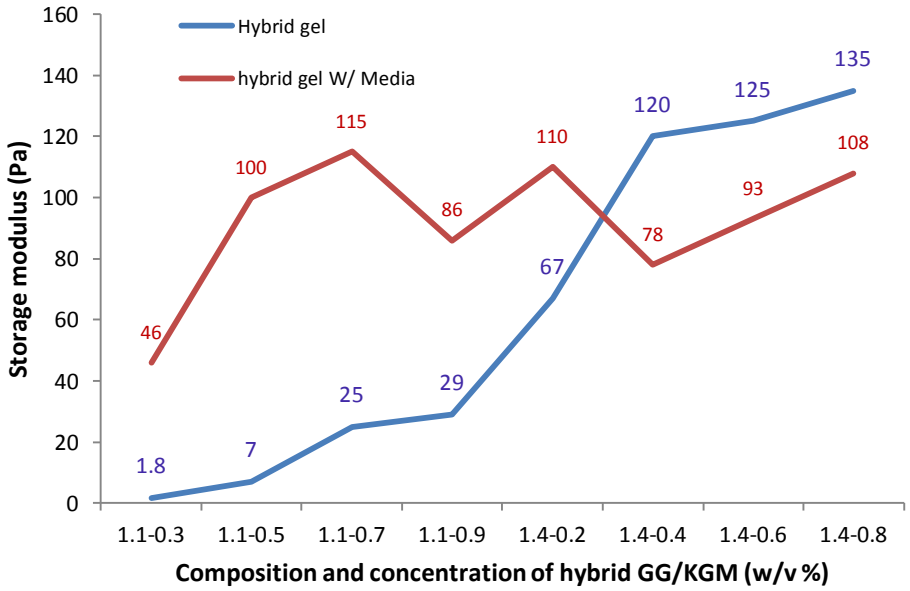


Figure 15: Storage modulus of GG/KGM hybrid gel with and without media
Blue (100µl hybrid GG/KGM) red (100µl GG/KGM sol mix with 100µl maintaining medium of NSCs)

Two series of hybrid hydrogel are made by using constant concentration of GG (1.1% or 1.4%) mixed with various concentrations of KGM. The blue line indicates GG/KGM gel without media, the storage modulus of hybrid gels based on constant 1.4% GG are higher than the storage modulus of those gels based on constant 1.1% GG. In each series, the storage modulus increases with the increasing concentration of KGM. In the red line of GG/KGM with media, the storage modulus changes differently. Except for GG/KGM of 1.1/0.3 gel, all other hybrid gels showed the similar storage modulus in the range of about 80~100Pa.

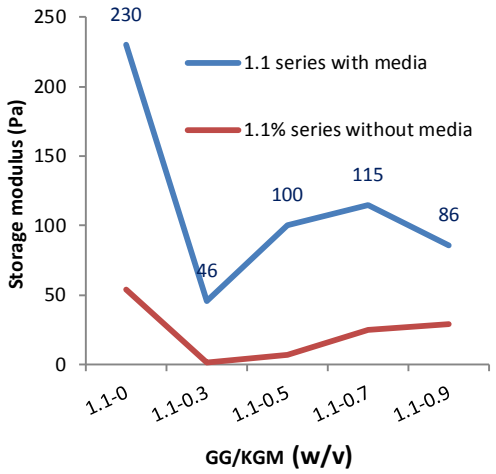


Figure 16: Storage modulus of 1.1% series GG/KGM hybrid gels
In presence of media (blue), storage modulus of 1.1% GG is higher than that of any 1.1% GG based hybrid gels by adding KGM

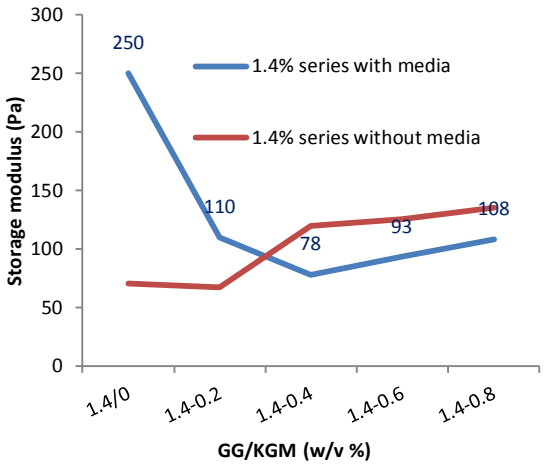


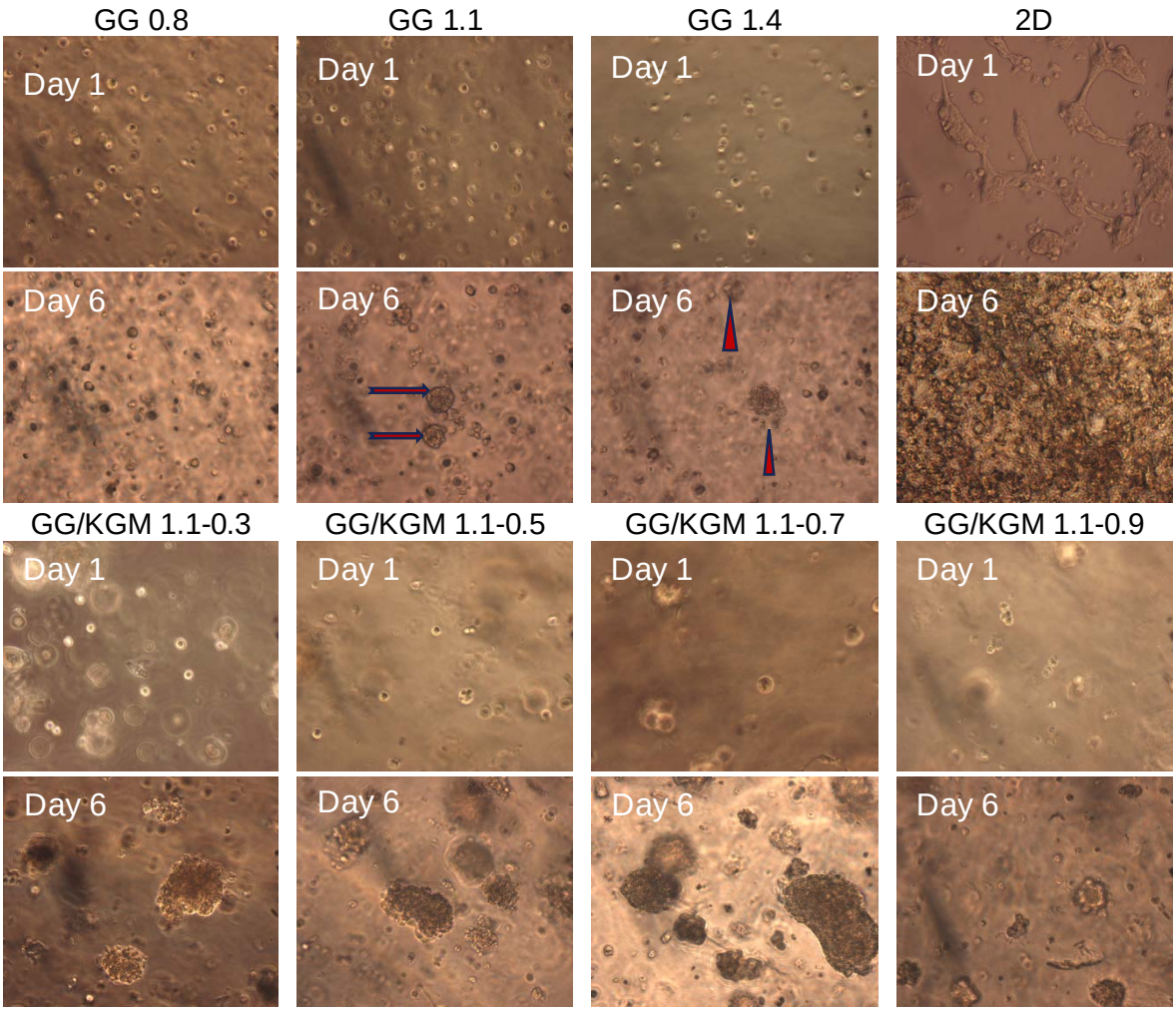
Figure 17: Storage modulus of 1.4% series GG/KGM hybrid gels
In presence of media (blue), storage modulus of 1.4 GG is higher than that of any 1.4% GG based hybrid gels by adding KGM

4.2 Cell Culture

4.2.1 Neural Stem Cells Culture

To check the suitability of 3D hydrogel on NSCs proliferation and migration, NSCs passage P7 (250K cells/well, $1.25*10^6$ cells/ml in 200µl gel and ratio of gel to media of 1:1) were seeded in 24 plates in different gels (GG 0.8%, 1.1%, 1.4% and hybrid GG/KGM in % w/v of 1.1/0.3, 1.1/0.5, 1.1/0.7, 1.1/0.9 1.4/0.2, 1.4/0.4, 1.4/0.6, 1.4/0.8). A 2D culture on matrigel coated surface was used as control to see the difference between 2D and 3D models. After seeding, the plates were checked by microscope to ensure that the cells distributed uniformly. Afterwards the plates were incubated under 37ºC and 5% CO_2 and media were changed each other day. The microscope pictures were taken on day 1 and day 6, the confocal pictures were obtained after 6 days cell culture following phalloidin and Dapi stain or live dead stain after cell fixing by 4% formaldehyde.

4.2.1.1 Microscope Pictures of NSC Culture



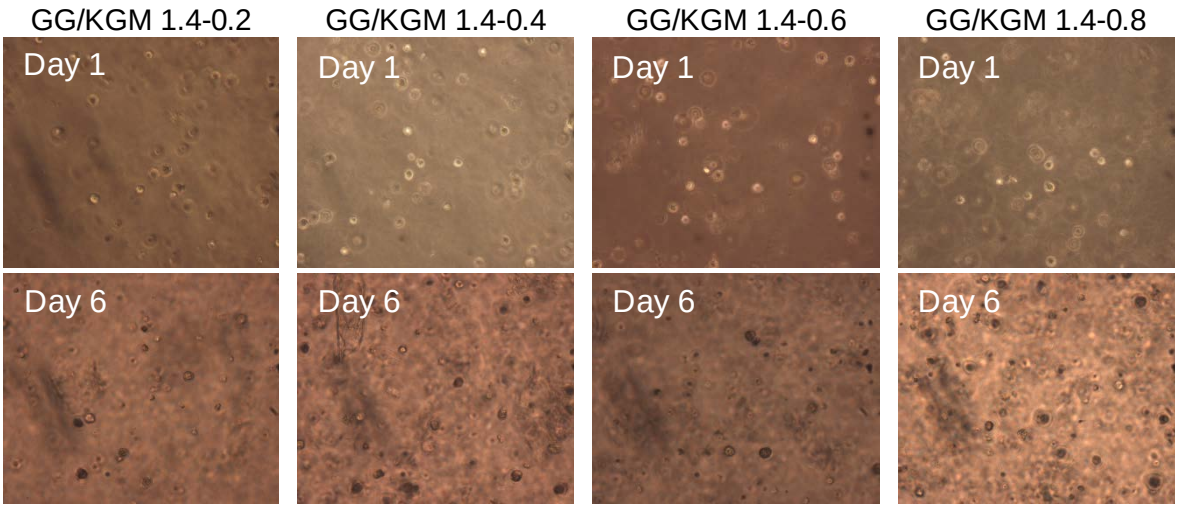
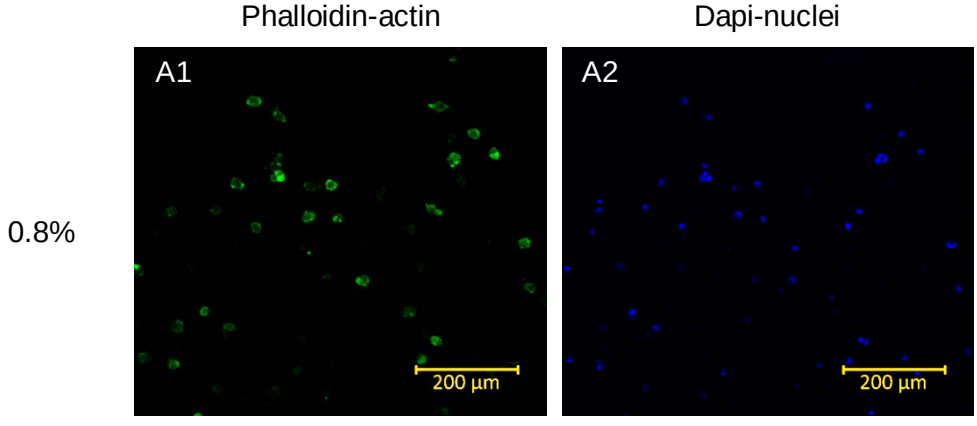


Figure 18: Microscope pictures of NSCs culture.
NSC P7 with 250K cells/well, $1.25*10^6$ cells /ml in 200µl gel, ratio of gel to media of 1:1, cell concentration in %w/V, magnification 20X.

Day one: Individual NSCs in all samples distributed evenly in gels and in round shape, except for NSCs in 2D control. Cells in the 2D culture attached the surface and the cells showed spreading shape different from those in 3D gel with round shape. Day 6: NSCs in 2D culture showed 100% confluent and formed a monolayer with fast proliferation. Comparing day 6 to day 1, cell proliferation can be observed in all samples. NSCs in 3D hydrogel showed different results. In pure GG gels, small neurospheres formed in 1.1% and 1.4% GG gels (blue arrow) whereas not the cases in 0.8% GG gel. Many big neurospheres formed in 1.1% GG based GG/KGM hybrid gels (constant 1.1%GG and various KGM). In 1.1/0.7 day 6, biggest sphere showed dark color in the middle may indicate cell death. Spheres in 1.1/0.3 on day 6 still showed bright color, this indicate the healthy sphere. No big neurospheres formed in 1.4%GG based GG/KGM hybrid gel (constant 1.4%GG and various KGM), where the NSCs kept individual cell or small spheres.

4.2.1.2 Confocal Scan of NSCs Culture



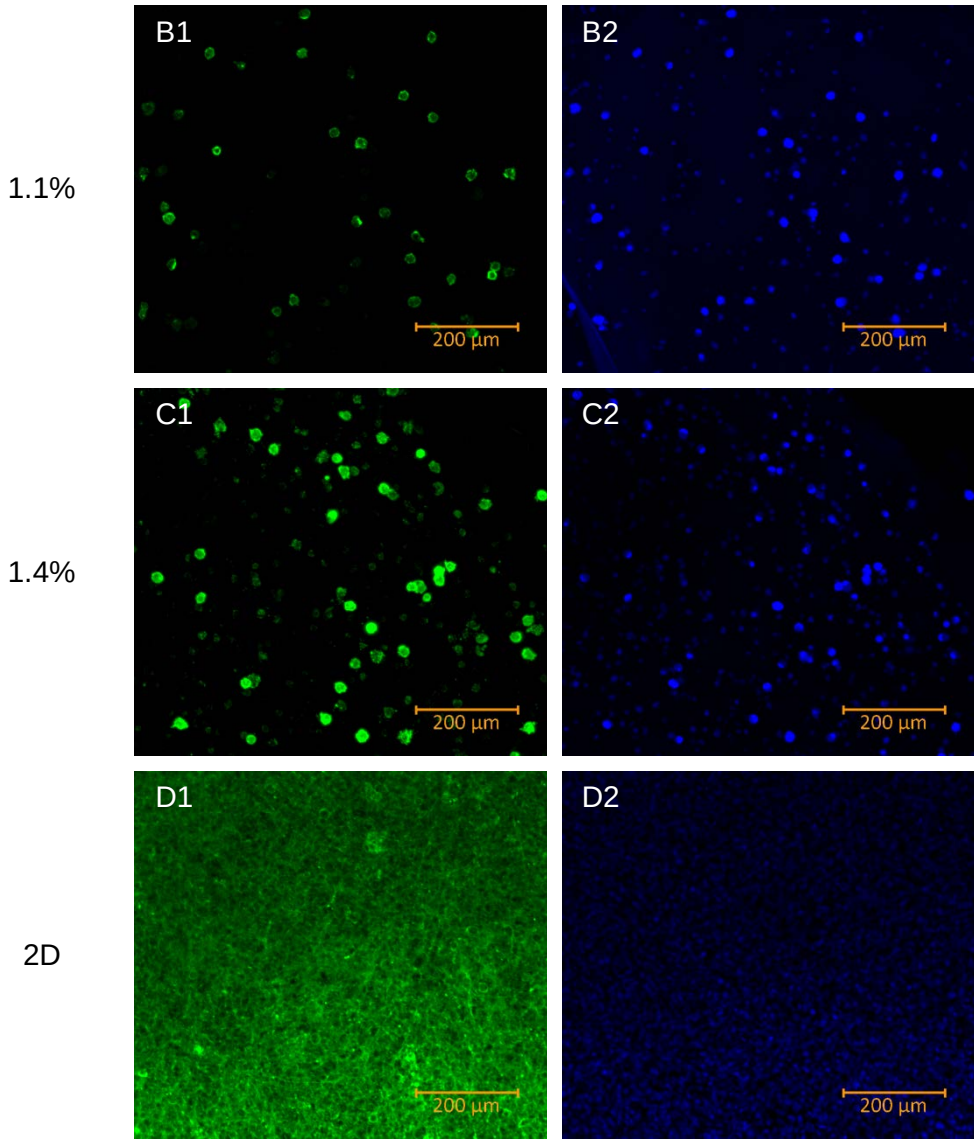


Figure 19: NSCs cultured in 3D GG gel and on 2D matrigel gel surface.
Green indicates actin stained by phalloidin. Blue indicates nuclei stained by dapi.
Magnification 10X

NSCs P7 were cultured on 2D (250K cells/well) and in 3D GG hydrogels (250K cells/ well, $1.25*10^6$ cells/ml in 200µl gel and ratio of gel to media of 1:1). After 6 days culture, in 2D control (D1 D2) the NSCs attached the surface of the matrigel coating and formed a monolayer with 100% confluent. However in 3D hydrogels (A-C), individual single cells distribute uniformly in the gels and kept round shape, very small cluster formed in 1.1% and 1.4% gels, and the most cells appeared in 1.4% gel. Less cells showed up in 0.8% (A2) GG hydrogel than that in 1.1% (B2) and 1.4% (C2) GG gel and there is no big different on proliferation between 1.1% and 1.4% hydrogel(B2 and C2).

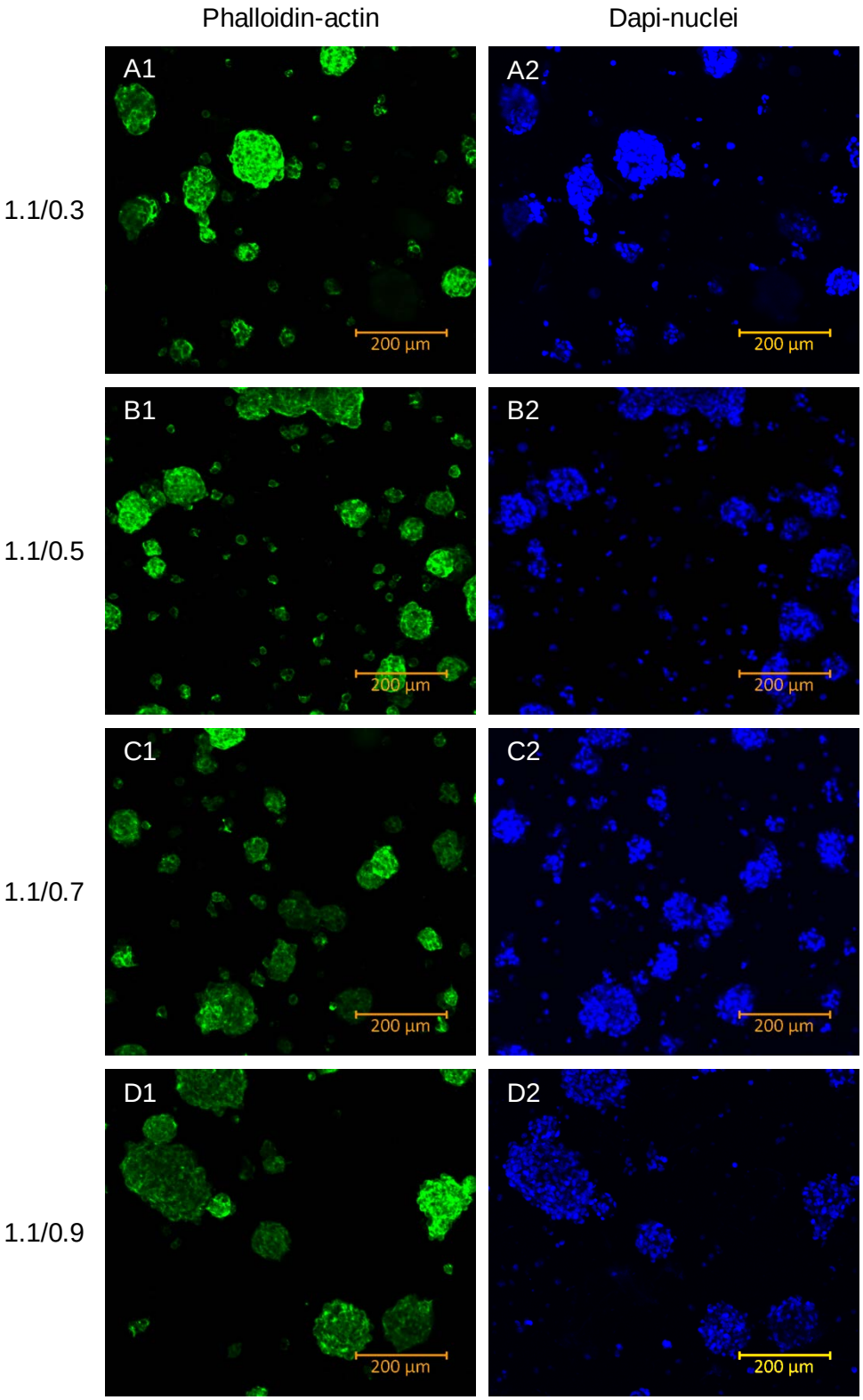


Figure 20: NSCs cultured in 3D 1.1% GG based GG/KGM hybrid hydrogels. Green indicates actin stained by phalloidin. Blue indicates nuclei stained by dapi. Concentration of gel GG/KGM is in % w/v. Magnification 10X

NSCs P7 were cultured 6 days in 3D hydrogels (250K cells/ well, $1.25*10^6$ cells /ml in 200µl gel and ratio of gel to media of 1:1).NSCs in all 1.1% GG based hybrid GG/KGM gels formed neurospheres (A-D), and more uniform spheres appeared in 1.1/0.5 (B1,B2)and 1.1/0.7 (C1,C2).

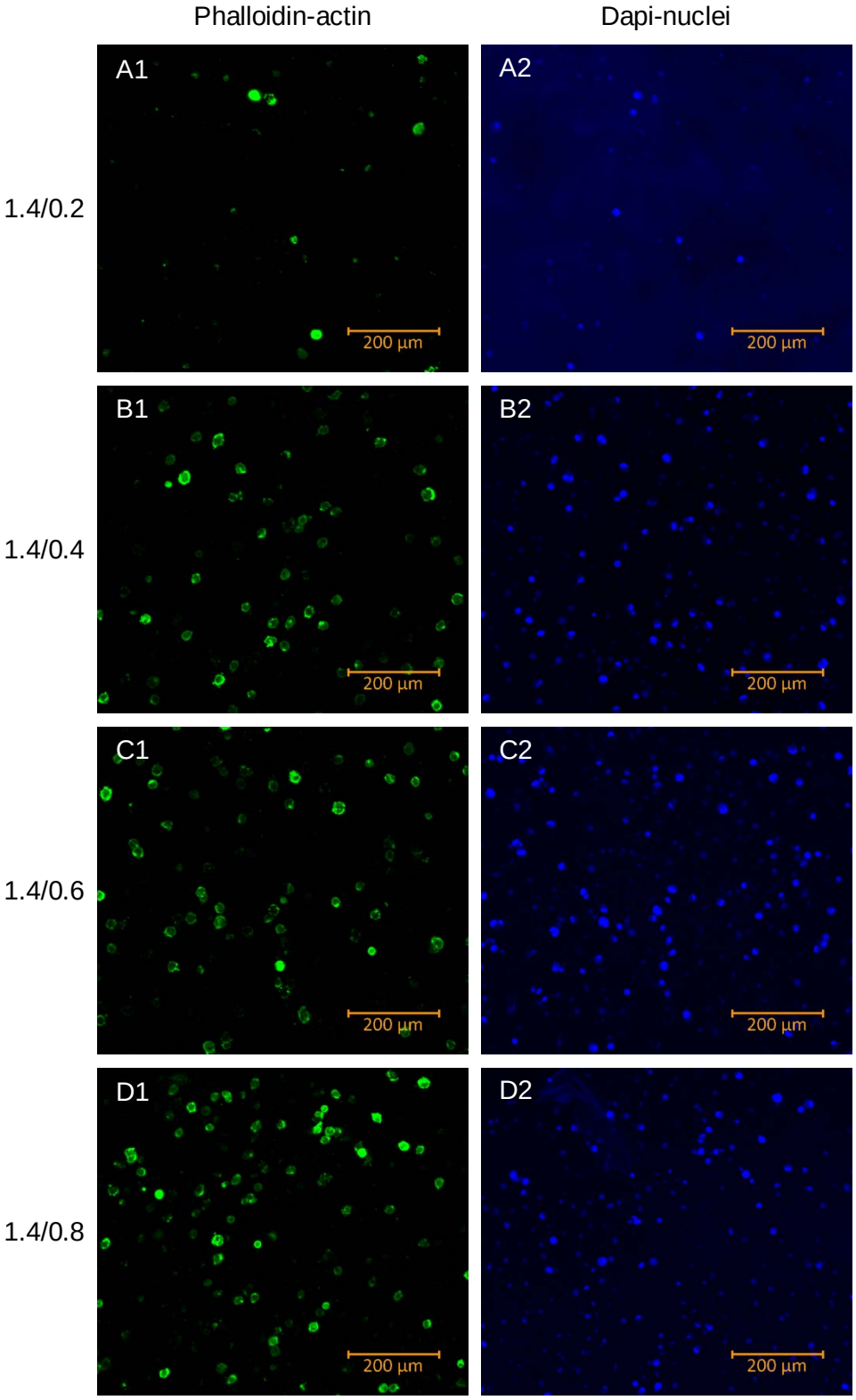


Figure 21: NSCs cultured in 3D 1.4% GG based GG/KGM hybrid hydrogel. Green indicates actin stained by phalloidin Blue indicates nuclei stained by dapi. Concentration of gel GG/KGM is in % w/v. Magnification 10X

NSCs P7 were cultured 6 days in 3D hydrogel (250K cells/ well, $1.25*10^6$ cells /ml in 200µl gel and ratio of gel to media of 1:1).No big neurospheres formed in all

1.4% GG based hybrid GG/KGM gels and all individual NSCs kept round shape. Less NSCs appeared in 1.4/0.4 (A2) and 1.4/0.6 (B2) and more NSCs showed up in 1.4/0.6(C2) and 1.4/0.8 (D2).

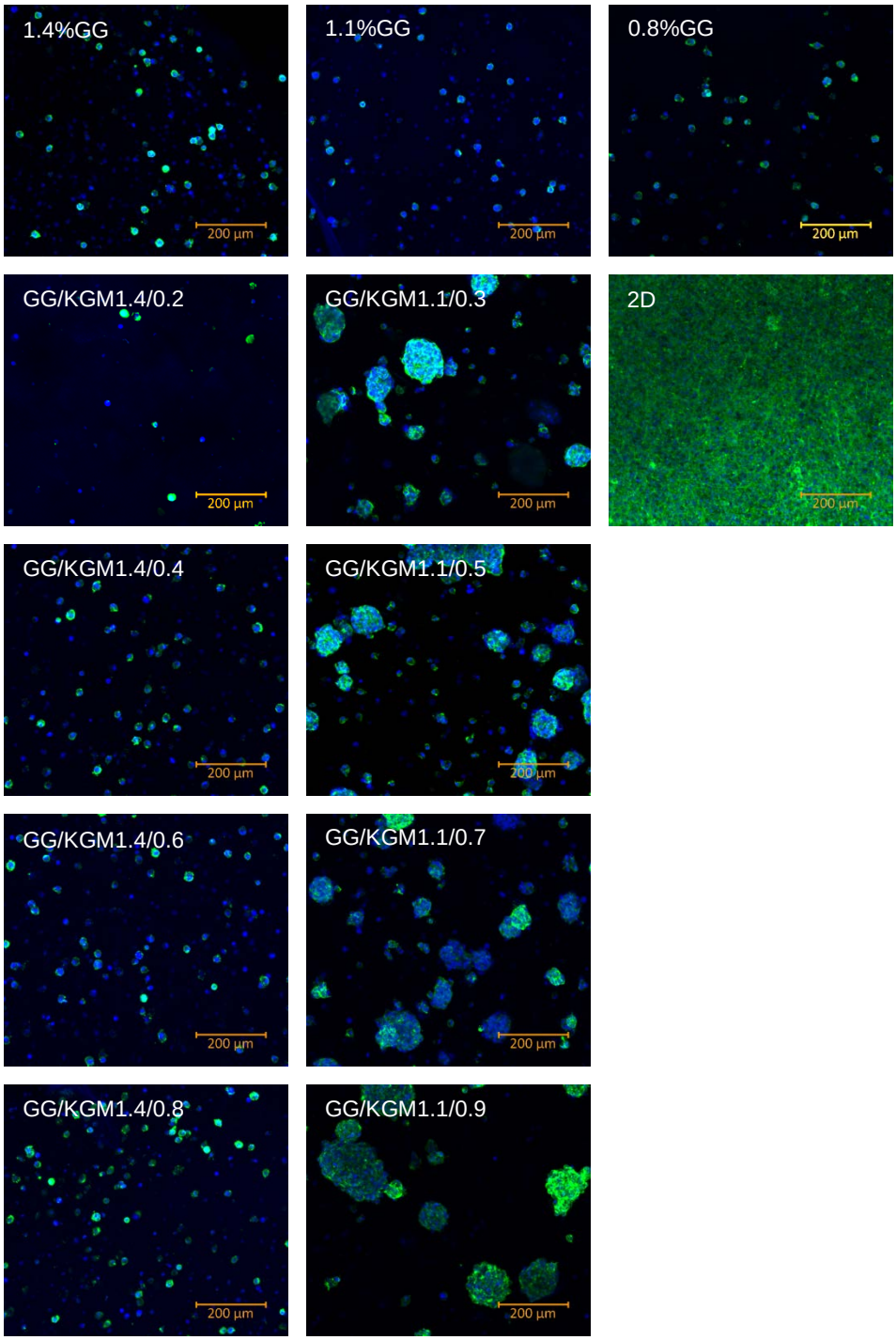


Figure 22: NSCs cultured in different 3D gels and 2D
Green indicates actin stained by phalloidin. Blue indicates nuclei stained by dapi. Concentration of gel GG/KGM is in % w/v. Magnification 10X

NSCs P7 were cultured 6 days in 3D gels (250K cells/ well, $1.25*10^6$ cells/ml in 200µl gel and ratio of gel to media of 1:1) and 2D. (1) NSCs in 3D showed individual round shape or spheres which are different as in 2D 100% confluent

monolayer. (2) 1.1%GG based hybrid gel showed big spheres whereas not the case in 1.1% pure GG gel alone (3). Except for less cells in 1.4/0.2 gel, there was no big difference of cell morphology and cell numbers in other 1.4%GG based hybrid gel comparing to 1.4% pure GG gel.

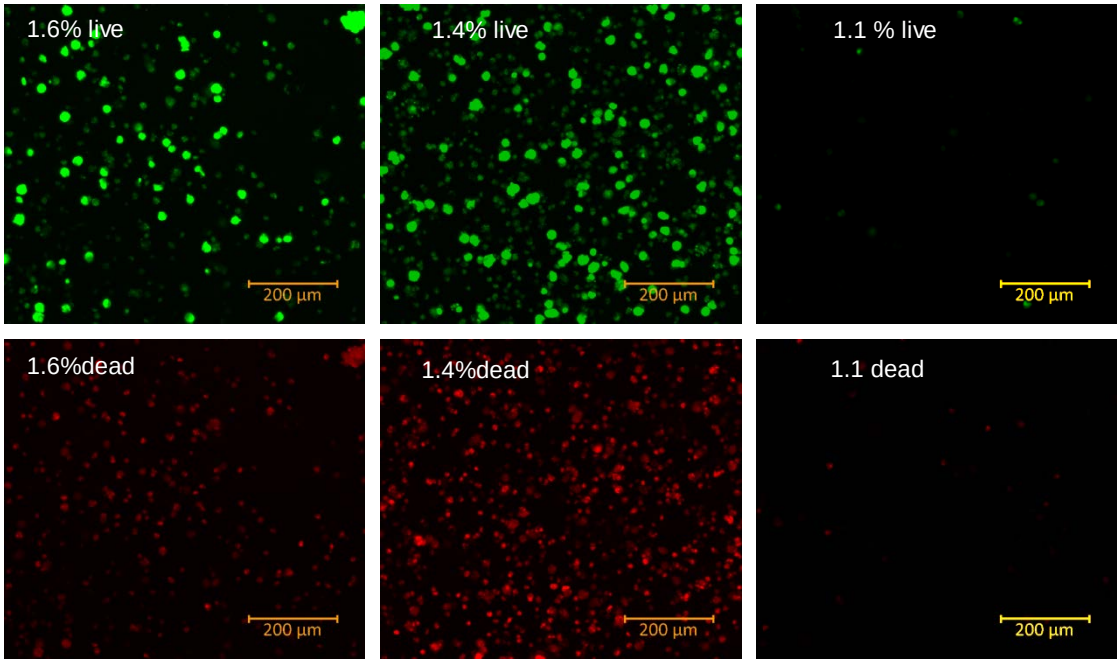


Figure 23: Live/dead stain of NSCs in GG gels

Green-fluorescent calcein-AM indicates intracellular esterase activity and red-fluorescent ethidium homodimer-1 indicates loss of plasma membrane integrity. Magnification 10X

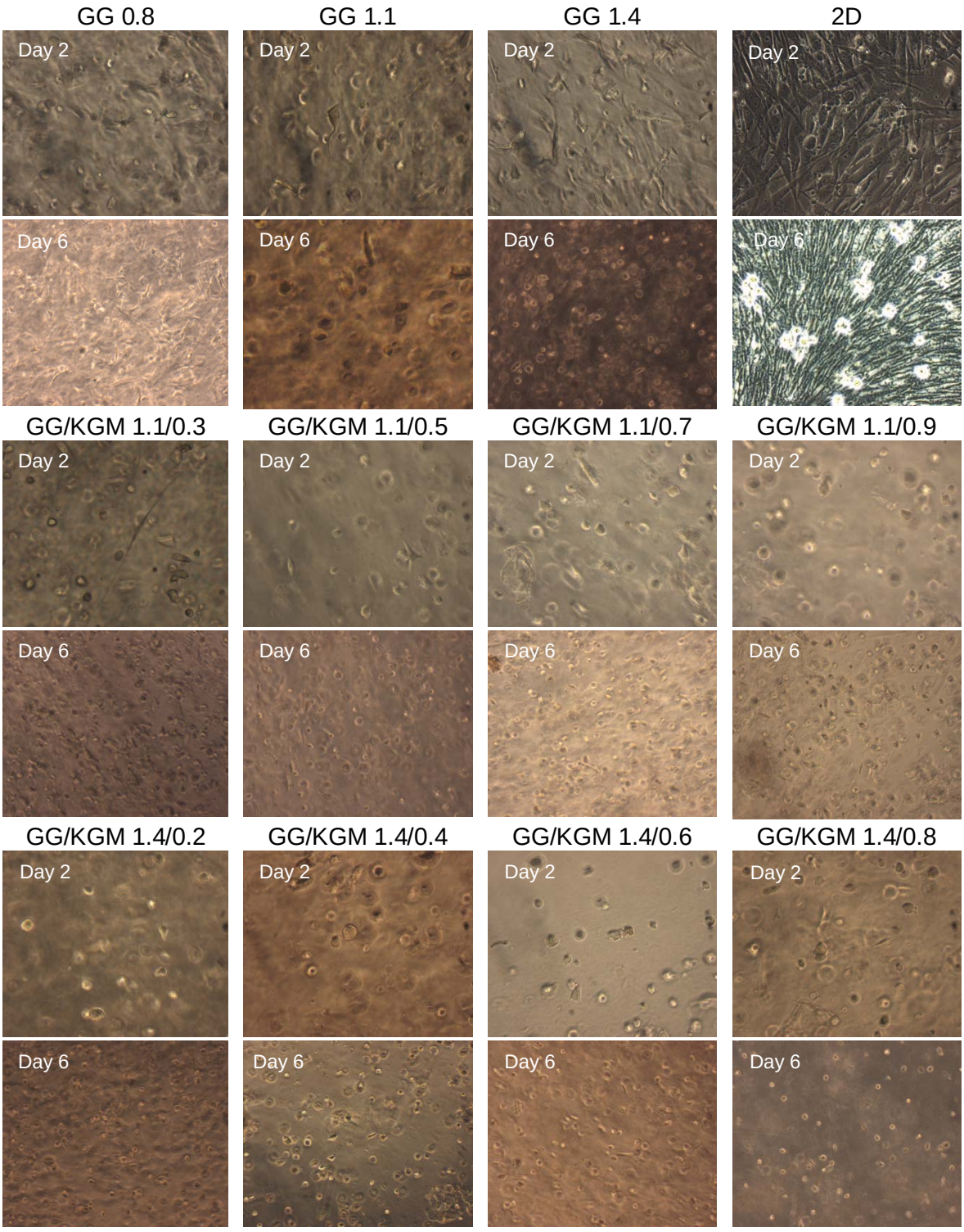
NSCs P7 were cultured in 3D GG hydrogel (500K cells/well, $2.5*10^6$ cells /ml in 200µl gel and ratio of gel to media of 1:1). After 6 days culture, live/dead stain were performed. The most cells appeared in 1.4% GG gel. This result may indicate that 1.4% GG hydrogel is suitable for NSCs growing.

4.2.2 Mesenchymal Stem Cells

MSC can develop to the neural lineage cells. To test the 3D hydrogel, MSC sub-passage P4 (250K cells/well,.$25*10^6$ cells /ml in 200µl gel and ratio of gel to media of 1:1) were seeded in 24 plates in different gels (GG 0.8%, 1.1%, 1.4% and hybrid GG/KGM in %w/v of 1.1/0.3, 1.1/0.5, 1.1/0.7, 1.1/0.9 1.4/0.2, 1.4/0.4, 1.4/0.6, 1.4/0.8, 0.6/0.6, 0.8/0.8 0/1.0). 2D culture was done as control to observe the difference between 2D and 3D models. After cell seeding the plates were checked by microscope to ensure that the cells distributed uniformly. Then the plates were incubated under 37ºC and 5% CO2 and media were changed each other day. The microscope pictures were taken on day 2 and day 6, the confocal pictures were obtained after 6 days cell culture following phalloidin and dapi stain

after cell fixing by 4% formaldehyde. Except for 2D and 1% konjac sample, all gels were moved into a new plate for confocal scan.

4.2.2.1 Microscope Pictures of MSC Culture



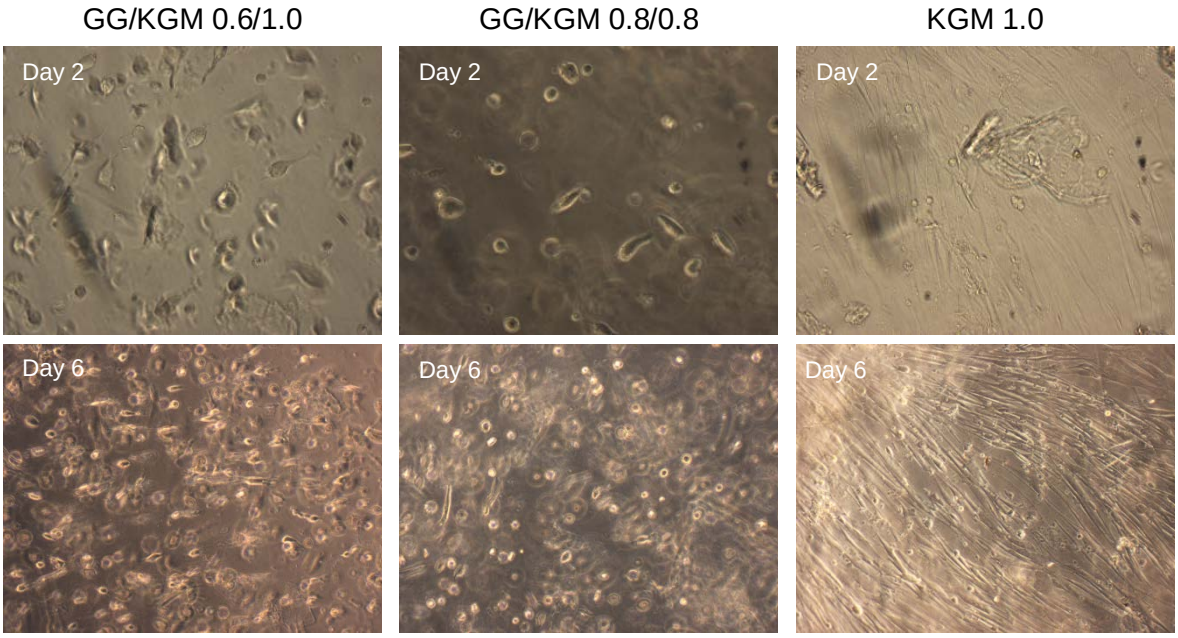
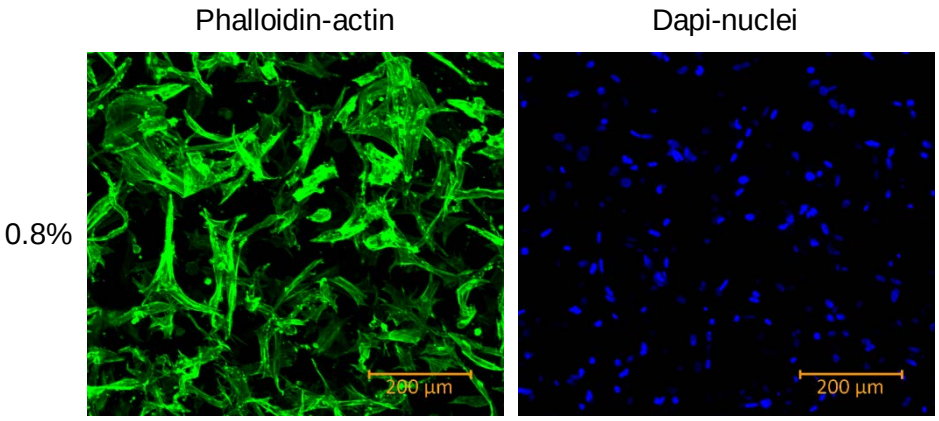


Figure 24: Microscope pictures of MSC in 3D gels (GG/KGM and GG) and 2D. Magnifications 20X. Concentration in w/v%

MSC P4 were cultured 6 days in 2D (250K cells/well) and in 3D GG hydrogels (250K cells/ well, $1.25*10^6$ cell/ml in 200µl gel and ratio of gel to media in 1:1). (1)Day 2: All individual cells appeared uniform distribution in the gels except for the monolayer formation on the 2D. In gels of 0.8, 1.1, 1.4, 1.1/0.7, 0.6/1.0, 0.8/08,1.4/0.8 some cells showed in ellipse-shape or spindle-shape. The most other cells are round. (2)Day 6: Comparing to the day 2, cell proliferation are obviously observed in all samples. In the gels of 0.8 GG and 0.6/1.0 more morphologic variations of cell shape appeared.

4.2.2.2 Confocal Scan of MSC Culture



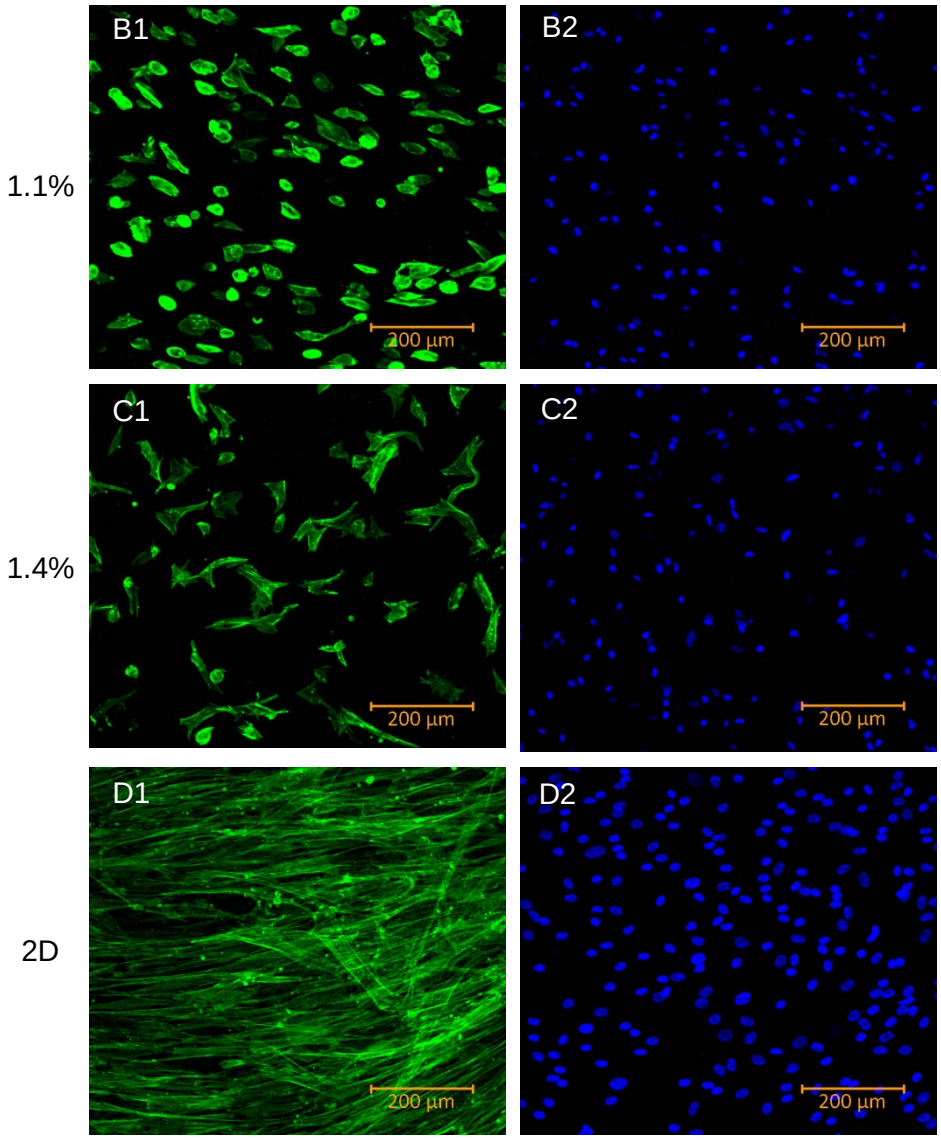


Figure 25: MSCs cultured in 3D GG gel and on 2D.
Green indicates actin stained by phalloidin. Blue indicates nuclei stained by dapi.
Magnification 10X

MSC P4 were cultured in 2D (250K cells/well) and in 3D GG hydrogel (250K cells/ well, $1.25*10^6$ cells /ml in 200µl gel and ratio of gel to media in 1:1). After 6 days culture, similar amount of cells have been detected in 3D GG gels of different concentrations (A2 B2 and C2). Morphology of MSC cells showed difference in 2D and 3D culture. Cells attached to the surface of plate and spread in 2D as a monolayer. In 3D gels, the most spreading cells appeared in 0.8% GG gel (A1) and some in 1.4% (C1) GG gels. More round cells appeared in 1.1% GG gel (B1).

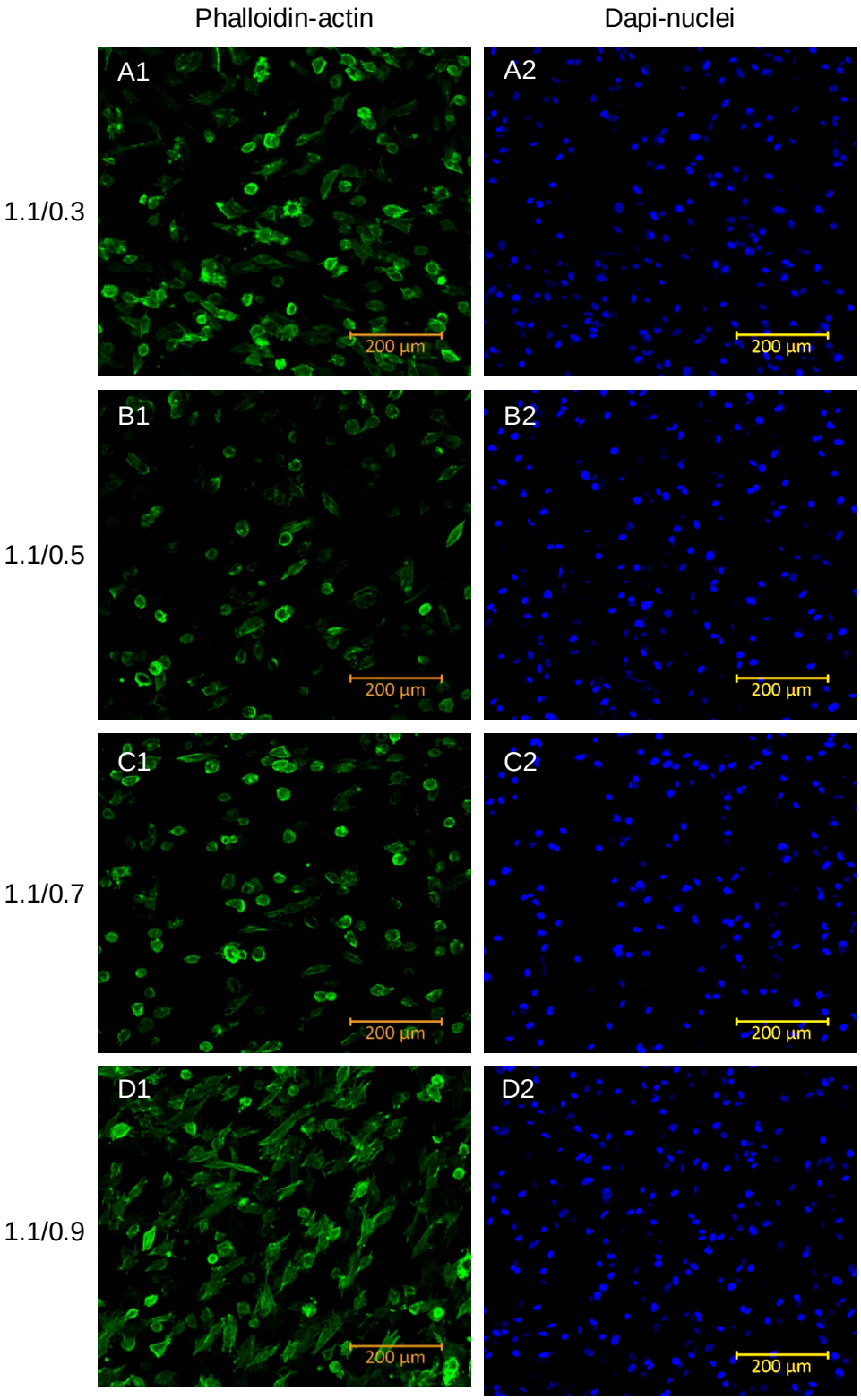


Figure 26: MSCs cultured in 1.1% GG based hybrid GG/KGM gels.
Green indicates actin stained by phalloidin. Blue indicates nuclei stained by dapi.
Concentration of gel is in %w/v. Magnification 10X.

MSCs P4 were cultured in 1.1% GG based hybrid GG/KGM gels (250K cells/ well, $1.25*10^6$ cells /ml in 200µl gel and ratio of gel to media in 1:1). After 6 days culture, MSC proliferate rates are similar in all 1.1% GG based hybrid gels (A2,

B2, C2 and D2). The most spreading cells were found in the gel of 1.1/0.9 GG/KGM (D1) whereas less in other gels.

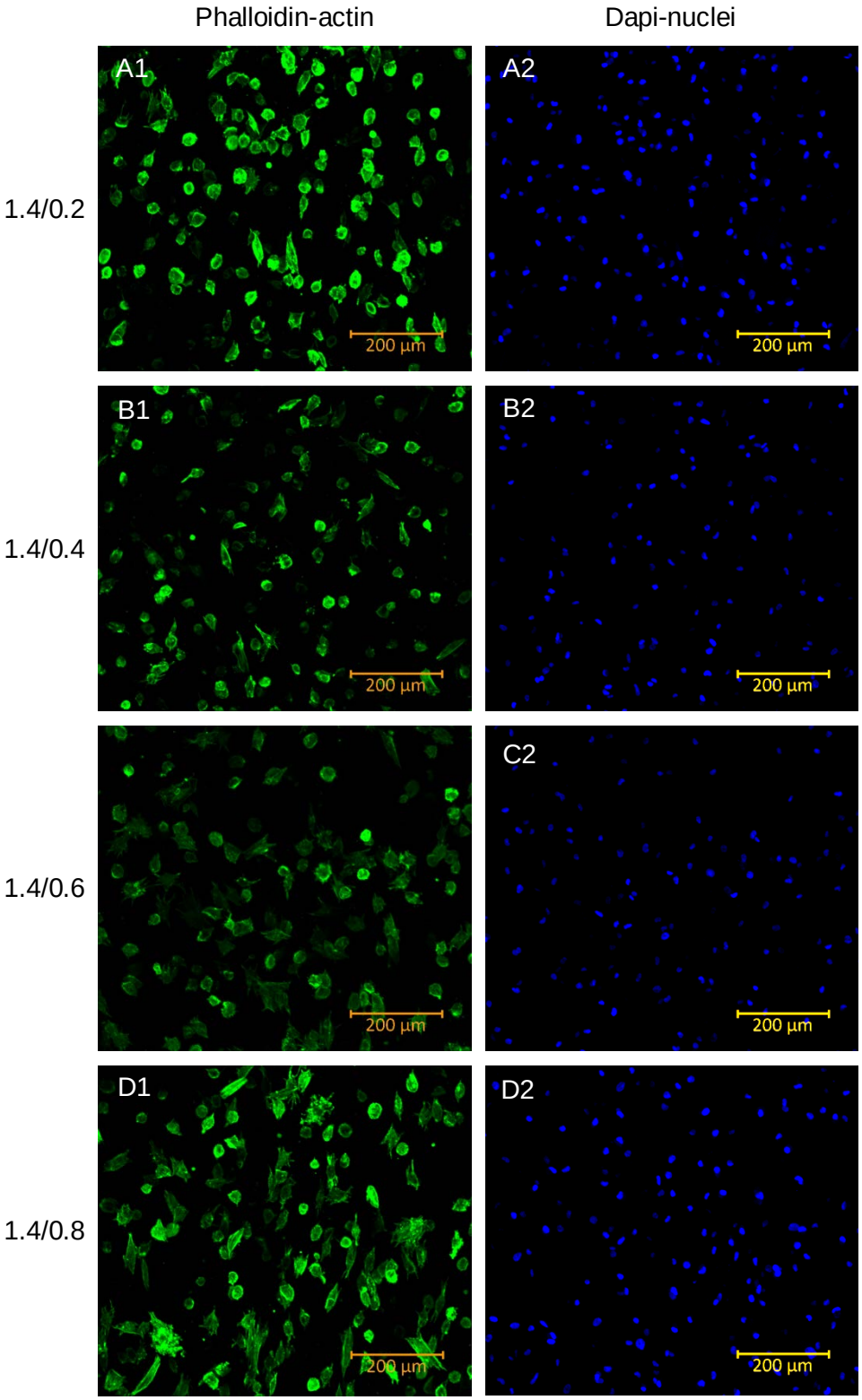


Figure 27: MSC cultured in 1.4% GG based hybrid GG/KGM gels.
Green indicates actin stained by phalloidin, blue indicates nuclei stained by dapi, concentration of gel in %w/v, magnification 10X.

MSCs P4 were cultured in 1.4GG based GG/KGM hybrid gels (250K cells/ well, $1.25*10^6$ cells /ml in 200µl gel and ratio of gel to media in 1:1). After 6 days

culture, MSCs proliferate rate is similar in all 1.4% GG based hybrid gels (A2, B2, C2 and D2) and showed similar morphologic variations, some cells slightly spread in 1.4/0.8 gel.

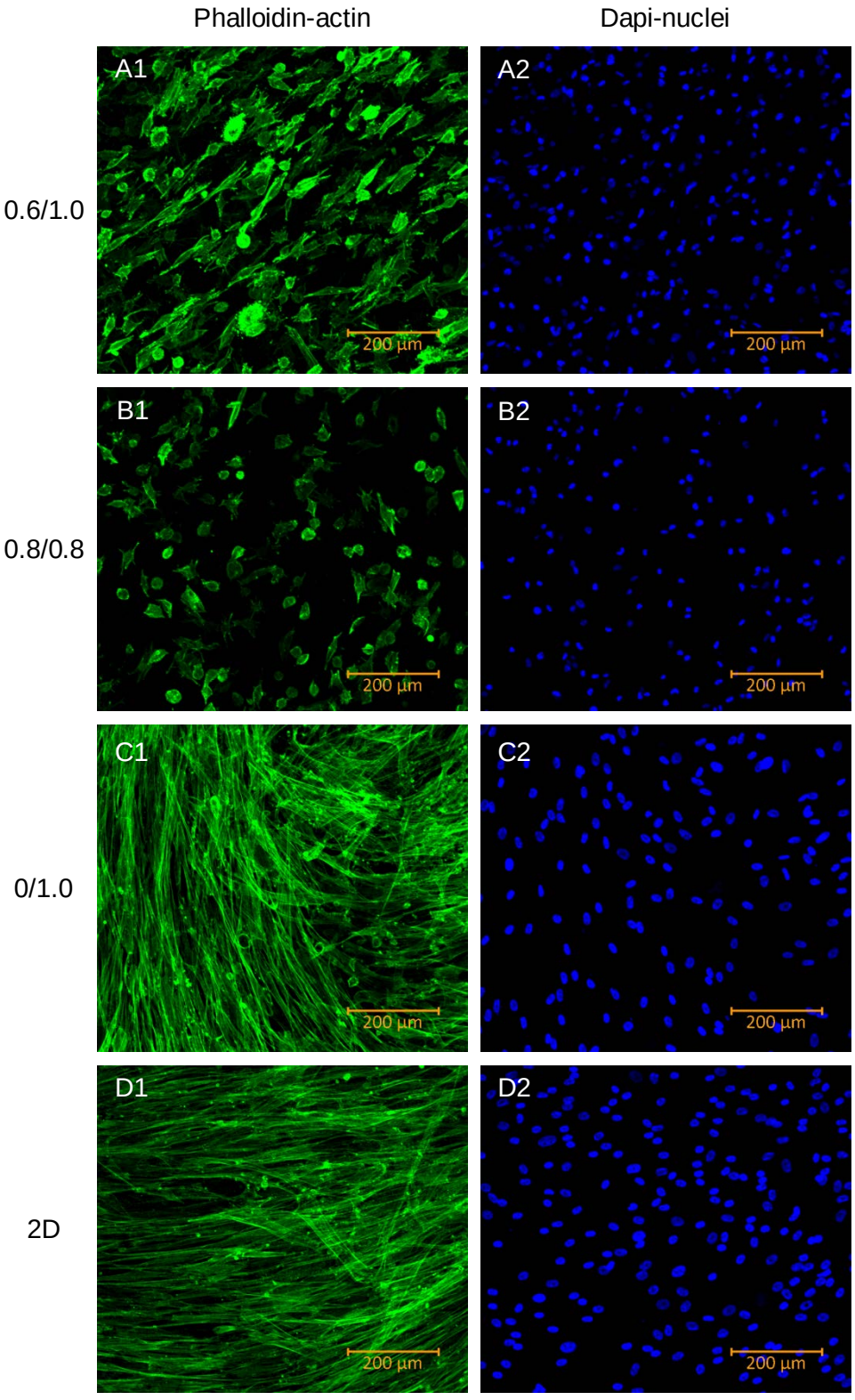
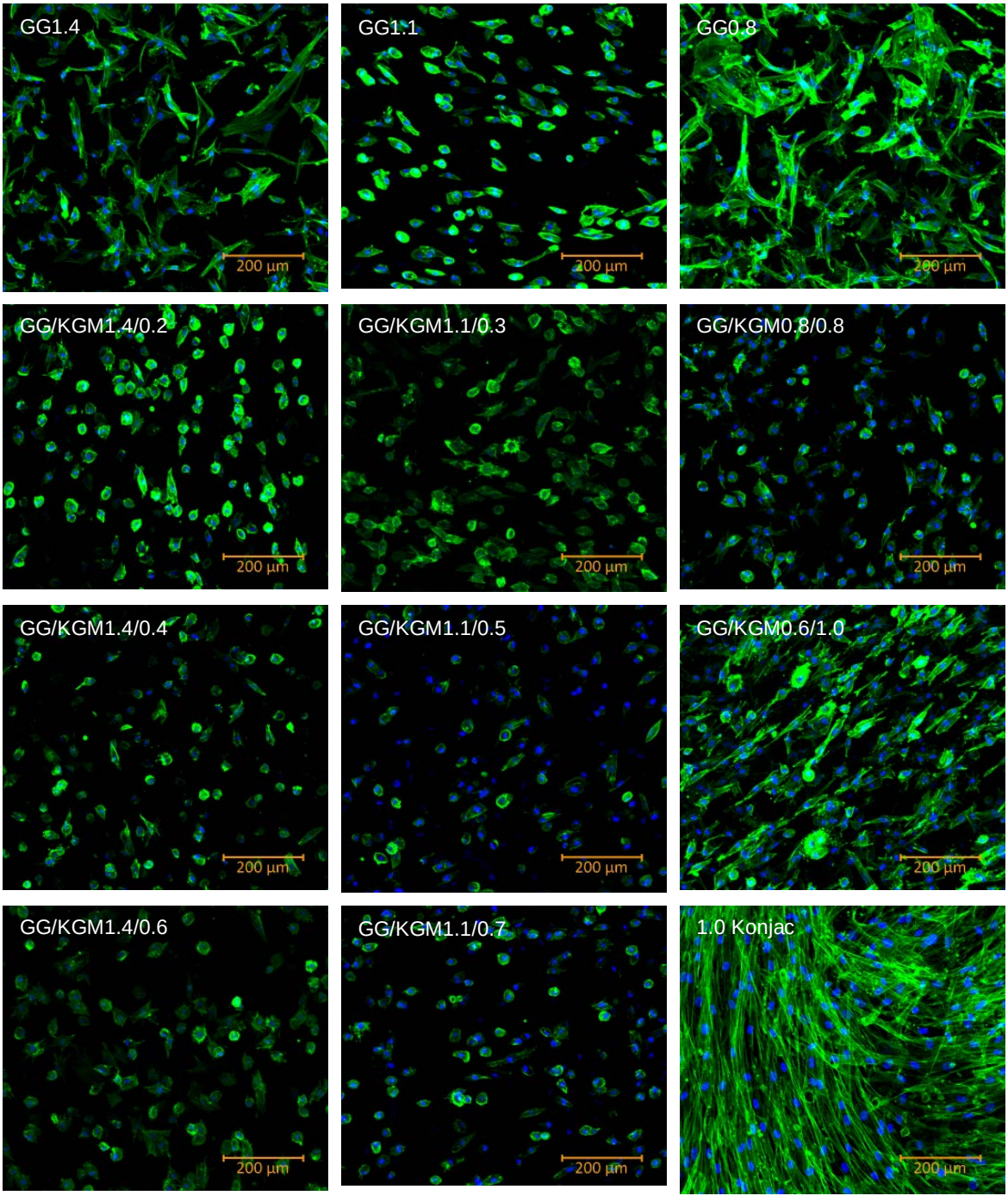


Figure 28: MSC cultured in 3D hybrid GG/KGM gels and 1% KGM.
Green indicates actin stained by phalloidin, blue indicates nuclei stained by dapi, concentration of gel in %w/v, magnification 10X.

MSCs P4 were cultured on 2D (250K cells/well) and in 3D GG hydrogel (250K cells/ well, $1.25*10^6$ cells /ml in 200µl gel and ratio of gel to media in 1:1). After 6 days culture, MSC proliferated better and spread more in GG/KGM of 0.6/1.0 gel (A1 A2) than in 0.8/0.8 GG/KGM gel (B1 B2). No gel formed when cells suspension mixed with the 1% konjac sol neither in room temperature nor under 37ºC. Therefore, MSC formed a monolayer in 1% konjac gel (C1 C2) same as in 2D (D1 D2).



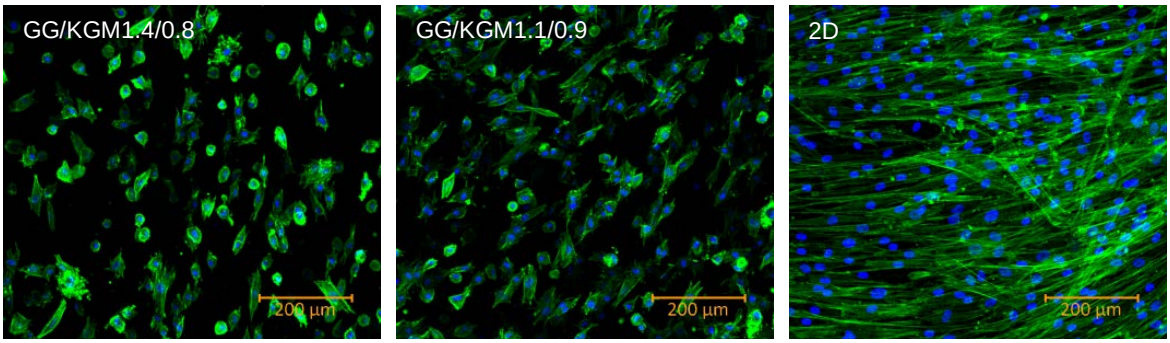


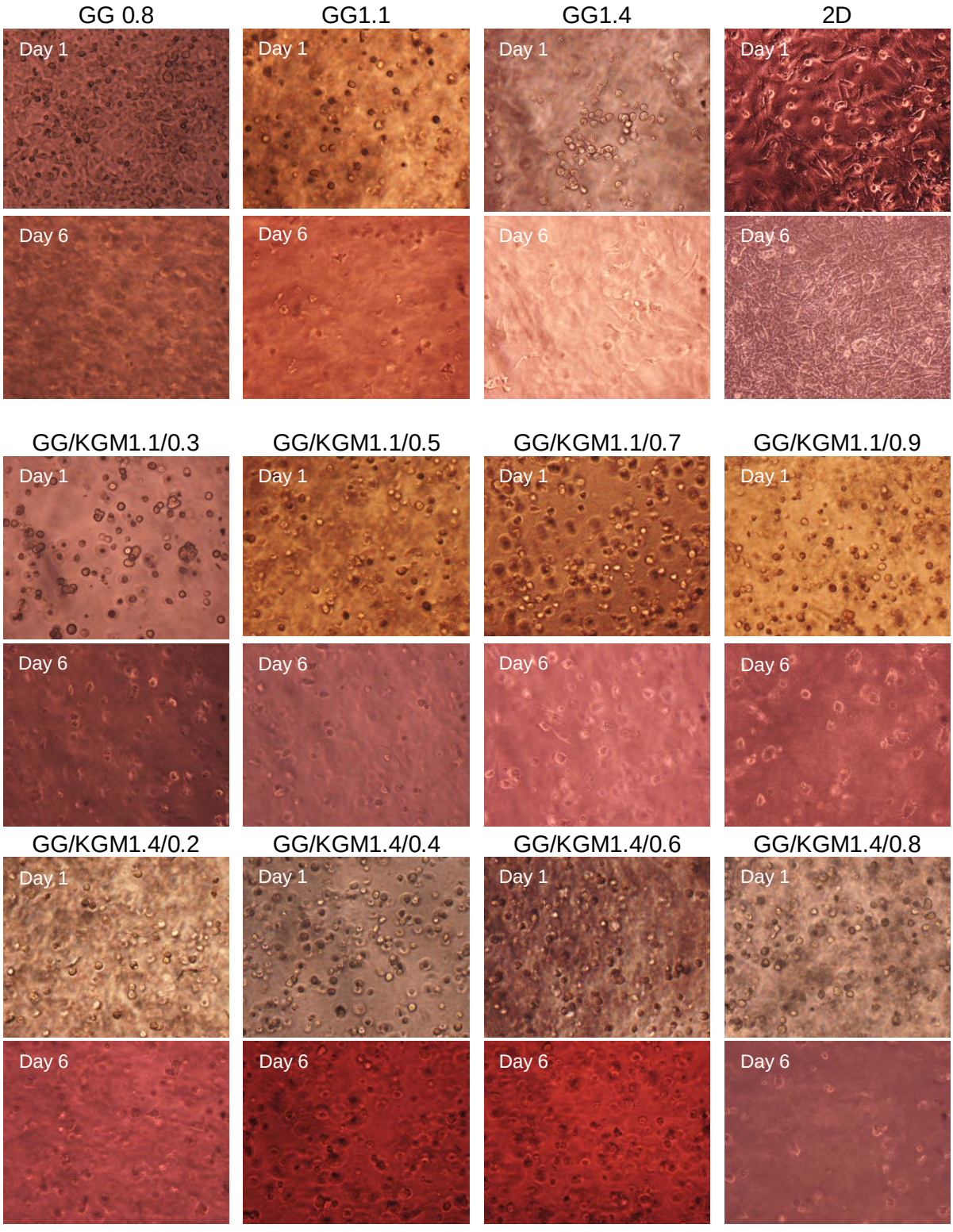
Figure 29: MSCs cultured in GG, GG/KGM, 1% konjac and 2D.
Green indicates actin stained by phalloidin, blue indicates nuclei stained by dapi, concentration of gel in %w/v, magnification 10X.

MSCs P4 were cultured in 2D (250K cells/well) and in 3D hydrogel (250K cells/ well, $1.25*10^6$ cells /ml in 200µl gel and ratio of gel to media in 1:1). After 6 days culture (1) 1% konjac did not form 3D gel by mixing MSC cell suspension and gel sol under neither room temperature nor 37 ºC, therefore in 1% konjac MSC formed monolayer same as in 2D. MSCs in both 2D and 1% konjac showed identical cell numbers and morphology which can be revealed by nuclei stain and actin stain.(2) Despite 1% konjac, all others formed 3D gels after mixing the cell suspension and gel solutions. In 3D gels, there is no significant difference on cell proliferation in 1.4GG based and 1.1GG based hybrid gels. More cells appeared in 0.6/1.0 GG/KGM gel. MSC spread the most in 0.8 GG gel and more in 0.6/1.0 GG/KGM. The morphologic difference in spreading cells in 3D and 2D can be obviously observed.

4.2.3 Meningeal Fibroblasts

Meningeal Fibroblasts sub-passage P4 (400K cells/well, $2*10^6$ cells /ml in 200µl gel and ratio of gel to media of 1:1) were seeded in 24 plates in different gels (GG 0.8%, 1.1%, 1.4% and GG/KGM in %w/v of 1.1/0.3, 1.1/0.5, 1.1/0.7, 1.1/0.9 1.4/0.2, 1.4/0.4, 1.4/0.6, 1.4/0.8, 0.6/0.6, 0.8/0.8 0/1.0). 2D culture was done as control to observe the difference between 2D and 3D models. After cell seeding the plates were checked by microscope to ensure that the cells distributed uniformly. Afterwards the plates were incubated under 37ºC and 5% CO_2 and media were changed each other day. The microscope pictures were taken on day 1 and day 6. Afterwards, the samples were fixed by 4% formaldehyde, stained by phalloidin and dapi, transferred into a new plate (except 2D and 1% konjac sample) and scanned by confocal camera.

4.2.3.1 Microscope Pictures of Meningeal Fibroblasts Culture



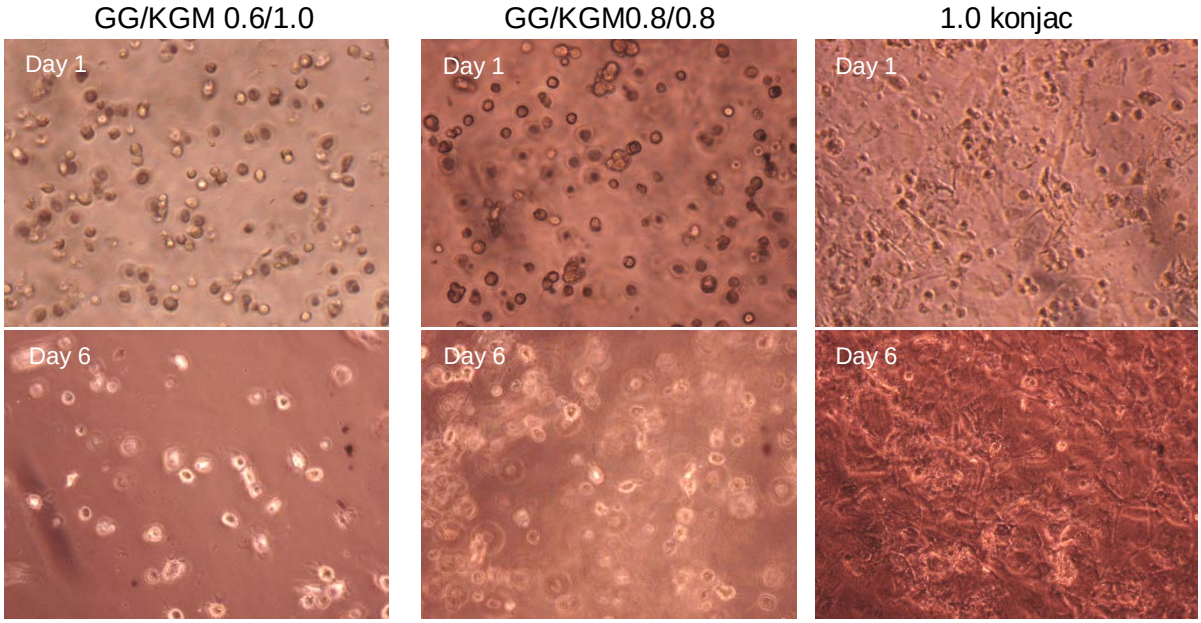


Figure 30: Microscope pictures of meningeal fibroblasts culture.
MF P4 with 400K cells/well, $2*10^6$ cells /ml in 200µl gel, ratio of gel to media of 1:1, gel concentration in %w/V, magnification: 20X

Day 1: All individual cells appeared uniform distribution in the gels and most cells are round except 30% confluent monolayer on the 2D and 1% konjac. Day 6: Comparing to the day 1, cell proliferation can be obviously observed in all samples. Monolayer formed in 1.0% KGM as that in 2D control with 100% confluent. In GG and GG/KGM gels, there is no big cluster formed but more morphologic variations appeared. No significant difference of cell numbers can be seen among all day 6 samples.

4.2.3.2 Confocal Scan of Meningeal Fibroblasts Culture

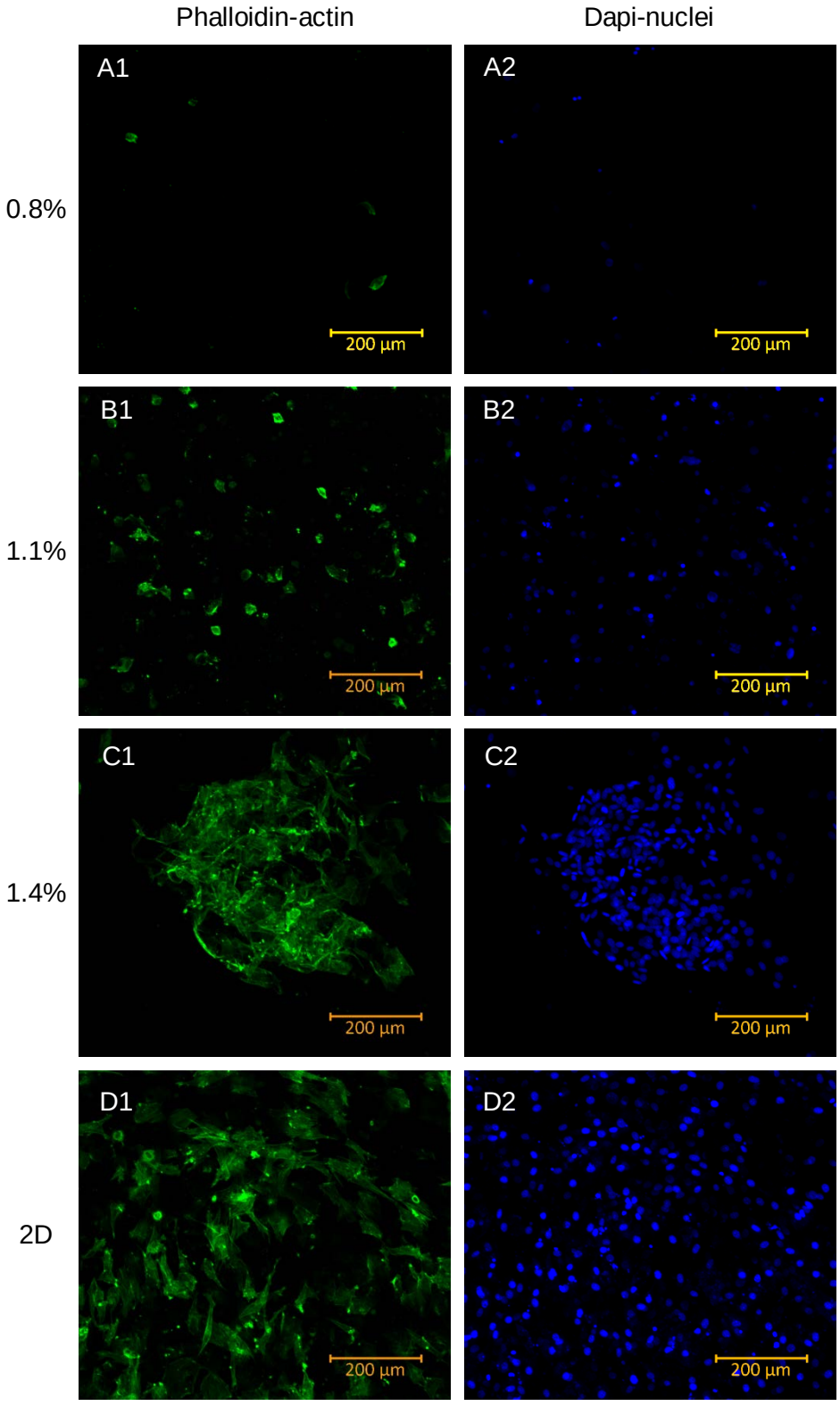


Figure 31: Meningeal fibroblasts cultured in 3D GG gel and on 2D.
Green indicates actin stained by phalloidin. Blue indicates nuclei stained by dapi.
Magnification 10X

MF P4 were cultured on 2D (400K cells/well) and in 3D GG hydrogel (400K cells/ well, $2*10^6$ cells /ml in 200µl gel and ratio of gel to media of 1:1). After 6 days culture MFs attached the surface of the well in 2D (D1 D2) and formed a monolayer with 80% confluent. In 3D GG gels (A-C), most cells appeared in

1.4GG gel (C2) with spreading 3D structure (C1). The less cells in 1.1 GG gel and fewer cells can be detected in 0.8 GG gel, MF kept round shape in 0.8 and 1.1 gel.

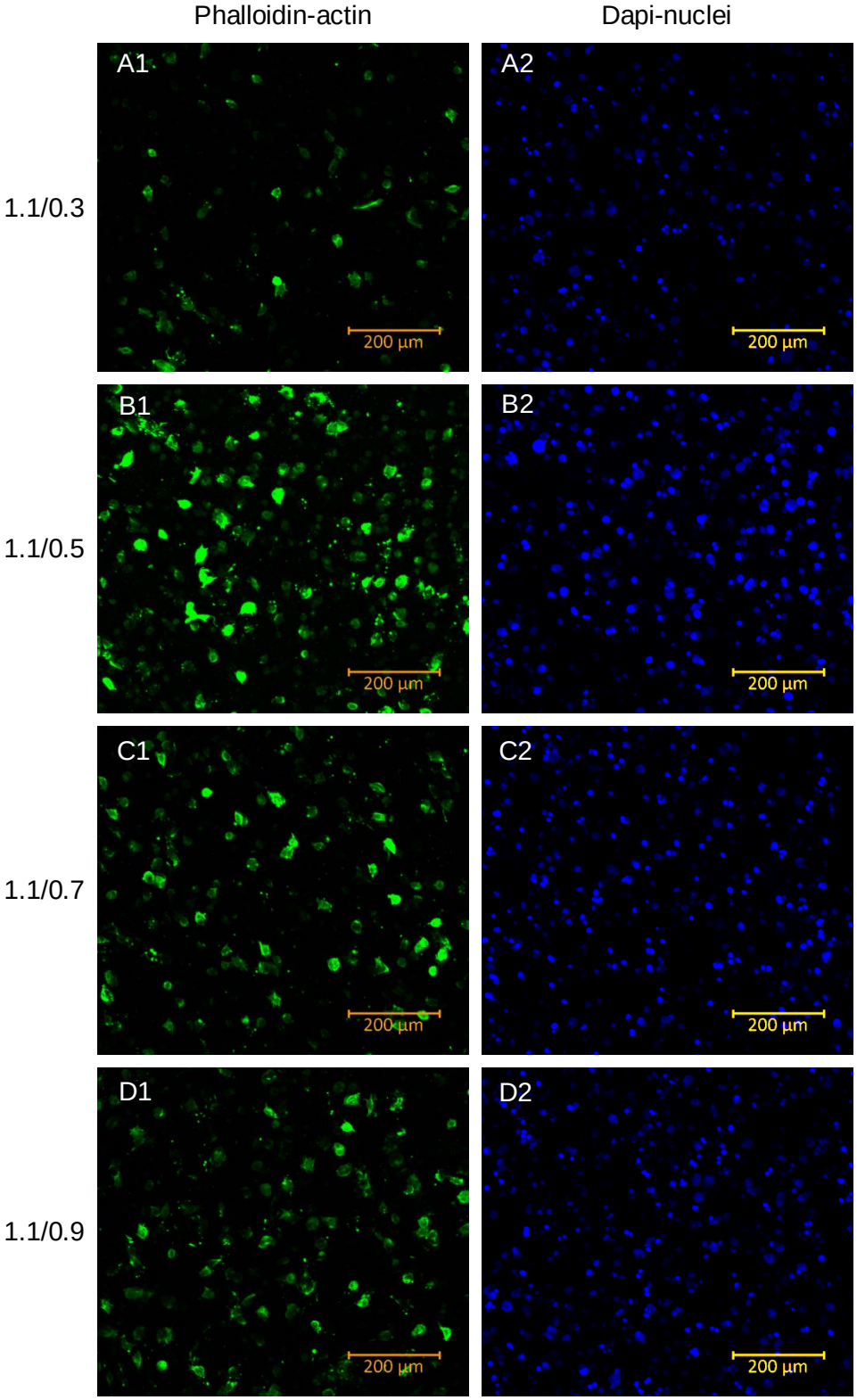


Figure 32: Meningeal fibroblasts cultured in 1.1% GG based GG/KGM gels. Green indicates actin stained by phalloidin. Blue indicates nuclei stained by dapi. Concentration of gel is in %w/v, Magnification 10X

MF P4 was cultured in 3D hybrid gels (400K cells/ well, $2*10^6$ cells /ml in 200µl gel and ratio of gel to media of 1:1). After 6 days culture, there is no significant

difference on cell proliferation and cell morphology among the samples in all 1.1 GG based GG/KGM gels exclude 1.1GG/0.3KGM, where showed less cells than others.

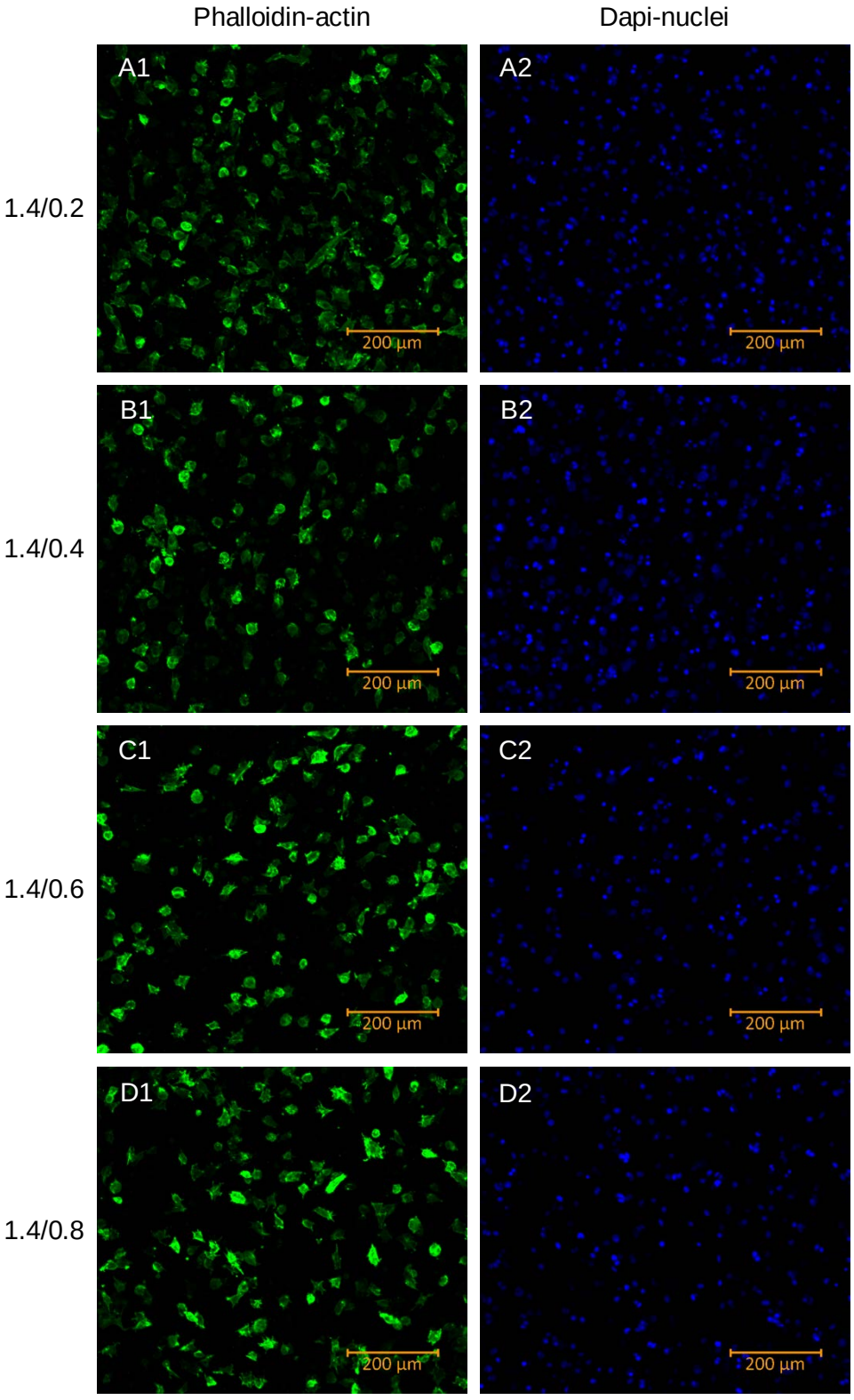


Figure 33: Meningeal fibroblasts cultured in 1.4% GG based GG/KGM gels. Green indicates actin stained by phalloidin. Blue indicates nuclei stained by dapi, Concentration of gel in %w/v, Magnification 10X

MF P4 was cultured in 3D hybrid gels (400K cells/ well, $2*10^6$ cells /ml in 200µl gel and ratio of gel to media of 1:1). After 6 days culture, there is no significant difference on cell proliferation and cell morphology among the samples in 1.4% GG based GG/KGM gels. Morphologic variations appeared in all samples, but cells didn`t spread as in 1.4% pure GG gels or 2D.

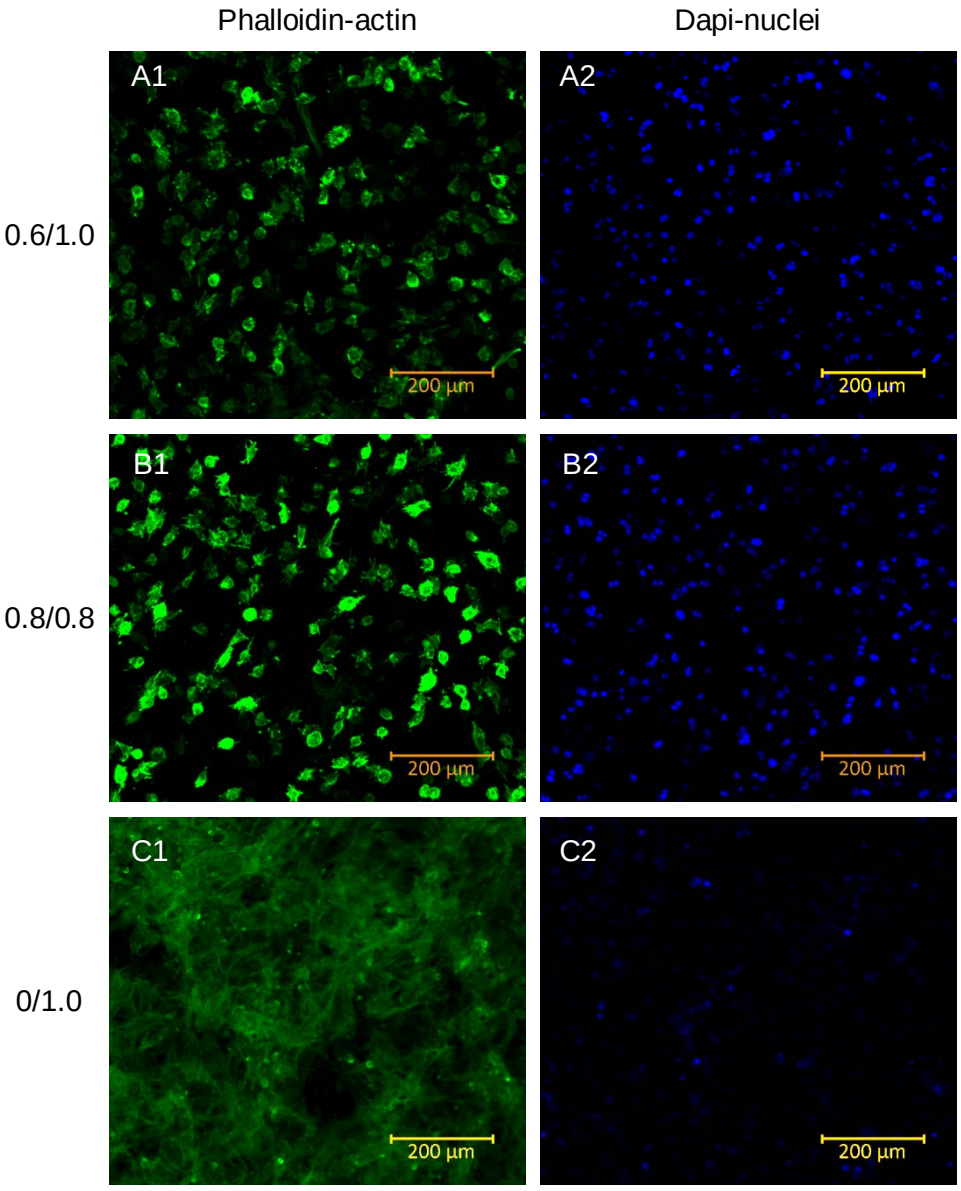


Figure 34: Meningeal fibroblasts in 3D GG/KGM gels and 1% KGM.
Green indicates actin stained by phalloidin. Blue indicates nuclei stained by dapi, concentration of gel in %w/v, Magnification 10X

MF P4 were cultured in 3D hybrid gels (400K cells/ well, $2*10^6$ cells /ml in 200µl gel and ratio of gel to media of 1:1) and in 1% konjac. There is no gelation occurred in 1% konjac after mixing the cells suspension with the gel solution. Thus monolayer formed in 1% konjac. No significant difference was found on cell proliferation and cell morphology between 0.8/0.8 and 0.6/1.0 GG/KGM hybrid gels. MF slightly spread in both gels.

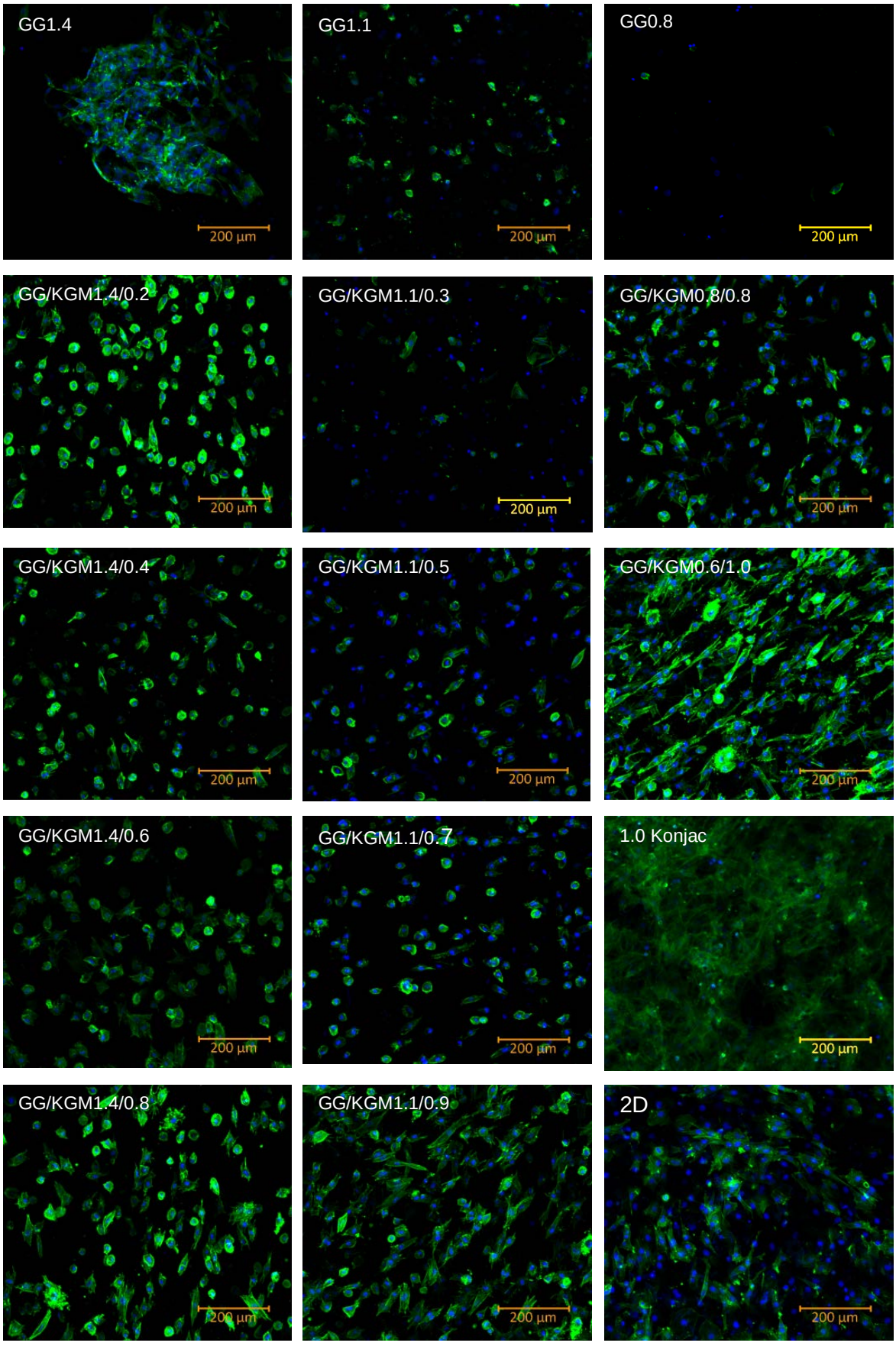


Figure 35: Meningeal fibroblasts in GG, GG/KGM, 1% konjac and 2D.
Green indicates actin stained by phalloidin. Blue indicates nuclei stained by dapi.
Magnification 10X

MF P4 were cultured in 3D hybrid gels (400K cells/ well, $2*10^6$ cells /ml in 200µl gel and ratio of gel to media of 1:1) and in 1% konjac as well as 2D. (1) In 1% konjac MF formed monolayer but showed difference in cell spreading as in 2D. (2) In GG gels, better proliferation and most cells spreading were found in 1.4% GG gel. Few cells were detected in 0.8 GG gel. (3) In 1.4% GG based hybrid gels, no significant difference of cell number could be observed among the hybrid gels. 1.4GG/0.8KGM showed slightly cell spreading. Comparing to 1.4%GG based hybrid gels, similar cell number with best cell spreading appeared in 1.4 pure GG gel .(4) Comparing to 1.1% GG gel with 1.1% GG based hybrid gels, cell proliferation in 1.1%GG was poorer than in most hybrid gels. No significant difference could be observed between 1.1%GG and 1.1GG/0.3KGM. More cells were found in gels of 1.1%GG with 0.5%, 0.7% and 0.9% KGM, but better cell spreading in GG/KGM of 1.1/0.9. (4) Comparing 0.8/0.8 to 0.6/1.0 hybrid gels, more cell spreading and cell numbers can be seen in 0.6GG/1.0KGM gel. (5) In all 3D gels, MF showed most cells spreading in 1.4%GG gel and higher cell number in 0.6GG/1.0KGM hybrid gel with slightly less cell spreading than 1.4GG gel.

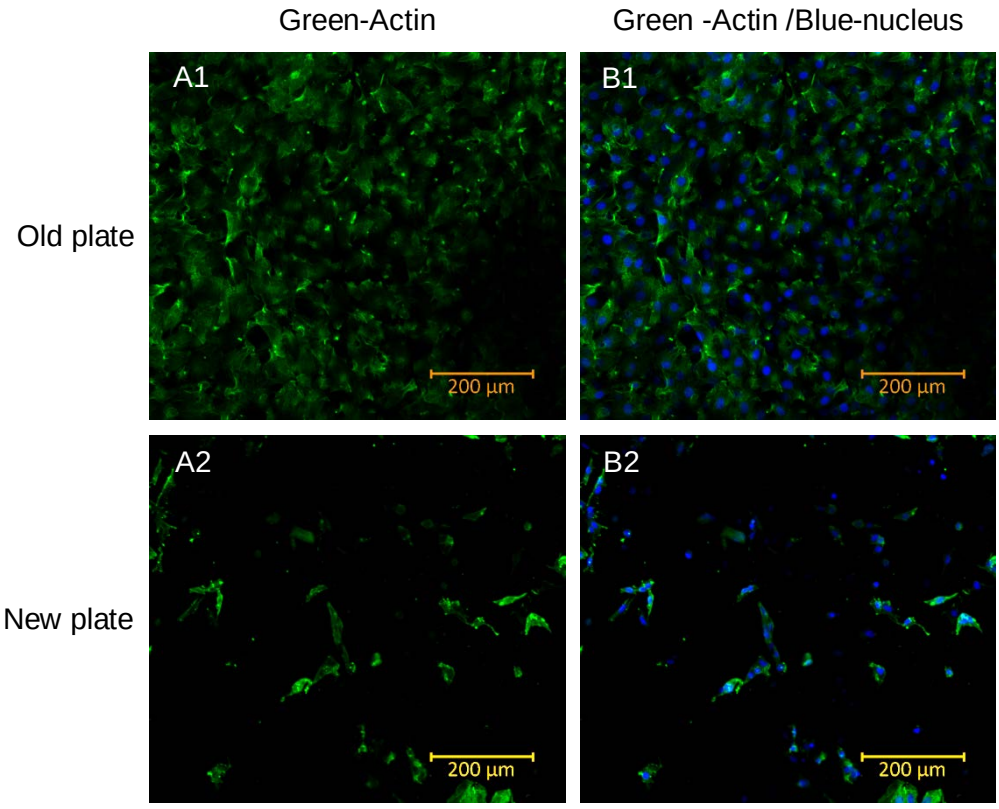


Figure 36: Meningeal fibroblasts on the edge of 1.4% GG gel

The edge of 1.4% GG gel in the original cultured plate was scanned. The MF showed spreading actin (A1) and more cells (B1) in this area. The gel was moved

to a new plate and scanned again. A2 and B2 showed fewer cells in the new plate in same area of gel. This revealed that MF could escape from the edge of gel and form a monolayer on the surface of the well.

5 Discussions

Hydrogel stiffness can influence the embedded cell on many cell activities, like adhesion, proliferation, migration, and differentiation. As we know, the stiffness depends on the type of materials, concentration of polymers, components ratio or concentration in the mixture, type of ions and their concentration, pore size and so on. Therefore, the optimal stiffness needs to be finely tuned by changing all factors which can influence the stiffness. Designing the stiffness of hydrogel need to consider not only the composition and concentration of polymers but also need to consider the used medium in cell culture.

In present work, the different conditions were used. Stiffness of GG was tested from 0.8% to 1.6% with and without media. The gel solution was prepared from 0.3% -1.7%. Above concentration of 0.8% under room temperature, the GG can form gels from soft 0.8% gel to hard 1.7% gel. Higher concentrated gels were excluded because it's stiffness in presence of media exceeding the range of ECM of CNS, e.g. gel of 100ul 1.7% GG mixing with 100ul media has stiffness greater than 1000Pa. In the GG gels, the stiffness of gel between 0.8%-1.6% is in the range of 3-250pa (blue line in Figure 14) and their storage modulus are dominant (Data not shown). With the increasing of the concentration of polymers, the storage modulus (G') is getting greater than the loss modulus (G") (Data not shown). For example G'/ G" of 0.8% GG is 3 and that ratio of 1.6% GG is 8, this indicates that the particles in the gels with higher concentration are strongly associated and more dense network formed. This is in compliance with other research result that stiffness of hydrogel increase with the increasing of the polymers concentration[130]. Below concentration of 0.8% the sol can form very soft gel which is below 100Pa in presence media, e.g. 0.8% GG in presence of media is only 100pa (Figure 14). Therefore, we focused on the gels with the concentration of 0.8%-1.6% since the stiffness in presence of media is between 130-673 Pa similar as the stiffness of ECM in CNS. To mimic the cell culture conditions, the 100µl gel solution with 100µl media (simulate cell suspension) were mixed by vortex to form gel, in which the actual polymer concentration was decreased 50% .The tested stiffness of those gels was in the range of 130-1050 Pa and was higher than the stiffness of pure gel without media. E.g. the stiffness of pure 1.4%GG is 70Pa. After 100µl 1.4% GG mixing with 100µl media, the final

concentration of polymers in this gel is 0.7%. The stiffness of this gel is 250 Pa, which is much higher than the 0.8%GG gel (3Pa) and 1.4% GG (70Pa) without media. This big change of the stiffness after mixing the GG with media resulted from the presence of the ions in the media, such as calcium chloride ($CaCl_2$), magnesium chloride ($MgCl_2$), magnesium sulfate ($MgSO_4$), potassium chloride (KCl), sodium bicarbonate ($NaHCO_3$), sodium chloride (NaCl) and sodium phosphate dibasic (Na2HPO4).This result revealed that during the stiffness design of cell culture system, not only the aspects of polymers concentration but also the ions [172, 173] in used medium for cell culture need to be considered, since the ions can significant influence the gel stiffness (Figure 14). To get the suitable gel, the stillness of the gel should be tested with the media or other solvent substance involved in the culture, for example PBS.

In the hybrid gels, the stiffness results reflect more complexity. 1% konjac alone couldn't form the gel under the normal culture conditions. Stiffness of pure GG gel of 1.1% and 1.4% GG in presence of media is higher than any hybrid gels based on this the concentration (blue line in Figure 16 and Figure 17), respectively. This may indicate that adding the KGM to GG can lead to a softer gel with loose structure. This is one of the assumptions of this study to make a hybrid gel of GG/KGM where hopefully the KGM with long fibre can change the microstructure of the GG network to get big pore size in order to increase the permeability. Ions influence on hybrid gels can be seen in Figure 15 , the blue line showed the stiffness of hybrid gels without medium. The 1.4% GG based hybrid series in general has higher stiffness than the 1.1% GG based series; this is in a compliance with the mechanisms that the GG is more responsible for the stiffness in the GG/KGM hybrid gel, which is mentioned in the introduction (Gellan Gum). The red line in Figure 15 shows the stiffness of the hybrid gels with media. The 1.1% GG based hybrid gels with medium have higher stiffness than the same series without medium, which occurs with the GG gel. But in the 1.4% based series, some hybrid gels with media have lower stiffness than those gels without media. For example, the stiffness of 1.4/0.8 GG/KGM with media is 108Pa whereas without media is 135Pa. This phenomenon may need further tests to understand more detailed mechanism to be able to design better gels.

To test the stiffness of hydrogel is first step to design a cell culture system. Only the hydrogels with similar stiffness as ECM of CNS may stimulate the adhesion

and proliferation of NSCs. Therefore, the 0.8%-1.6% GG gel (100-700Pa) and all hybrids gels (50-150Pa) which are in the range of stiffness (less than 1000pa) of ECM of CNS could be suitable for NSCs culture system. But 1.6% GG gel was not often used in cell culture in present work since the 1.6% GG gels is not easy to get the sol with simply shaking under room temperature, whereas 1.4% GG occurred. As we know that the optimal matrix stiffness varies among different cell types, three different cells (NSCs, MSCs and brain tissue isolated MFs) in this study are used for cell cultures. Both proliferation and morphologic change of the cells were observed.

NSCs appeared different proliferation and phenotype in 3D gels. In GG gels, NSCs show the best proliferation rate in 1.4% GG gel with stiffness about 250Pa instead of softer gel of 0.8% of 130Pa (Figure 14). Live/dead stain also suggested the most live NSCs in 1.4% GG gel (Figure 23). Most cells in this gel are round, only small spheres are formed. This may suggest that the pore size of the gels may not optimal for cell migration or the adhesion of NSCs to GG gels is generally weak. NSCs proliferate better in the 1.1% GG based hybrid gels comparing to 1.1%GG gel. Big neurospheres formation may reveal better cell migration and proliferation in these gels. The further tests of pore size need to be done to get the clue of the optimal diameter of GG or GG/KGM hybrid gels for NSCs. To enhance the cell adhesion in order to improve cell proliferation, GG gels or GG/KGM hybrid gels can be modified with some adhesion peptides or with adhesion-favorable components. For example, hydrogels composed of collagen and laminin-derived cell adhesive peptide can enhance the survival of embedded neural cells [95]. Self-renewal is essential for NSCs to maintain their population in an undifferentiated state, which is critical for cell cultures. CAMs play a critical role during the cell developing process, since CAMs are the bridging molecules between extrinsic signals from neighbor cells or the ECM and intracellular transcriptional regulation. Therefore, to control NSCs self-renew and keep NSCs properties, CAMs can be considered as additional molecule to improve the culture system. L1, for example, a subfamily protein of CAMS, has been reported to play an important role in neuronal survival and migration as well as neurite outgrowth and extension, axonal guidance and synaptic plasticity in vitro and in vivo [174]. Since the neighboring cells form an important component of the static mechanical environment sensed by a given cell, therefore the cell-sense may play a role in 3D culture systems. To test the influence of cell-cell interaction on cell survival and

proliferation, different cell seeding density to change the distance of cell-cell can be used in next experiment. Co-culture with other cells which are the nature neighboring cells of NSCs may help to get more hints to culture the NSCs. Due to the special components, to understand ECM of CNS better also can help the design of cell culture. Proteoglycans, which present in the extracellular microenvironment and on the cell surface, can bind diverse extracellular factors, including signaling molecules, membrane proteins, and components of the extracellular matrix. Proteoglycans play a vital role in cell–cell and cell–ECM signaling in the CNS. Tenascin C (TNC) is an extracellular matrix glycoprotein that is highly expressed by NSCs located in the brain and spinal cord during development and in the adult. Glycan binding proteins, divided broadly into lectins or glycosaminoglycan binding proteins, are thought to mediate many of the biologic functions of glycans. Cell adhesion to ECM is a critical step, the major cell surface receptors that mediate this interaction are the integrins. The basal lamina, enriched in laminin and collagen, helps to regulate adhesion of NSCs to the ECM. To understand the interactions between NSCs and niches and to use these substances in cell culture may influence NSC adhesion, migration, cell–cell communication, and cell survival. Peptide modified GG and co-culture with olfactory ensheathing glia cells demonstrated significantly greater survival and proliferation relative to monocultures of NSPCs [145].

MSCs are multipotent self-renewing cells derived from adult bone marrow. They can differentiate to osteoblasts, chondrocytes and adipoicytes and also trans-differentiate to neural lineage [175, 176]. Easy isolation and high expansion potential as well as the ability to differentiate into multiple lineages make them widely used in cell therapy [177]. Marrow stromal cells have showed to form guiding strands in the injured spinal cord and promote recovery in a rat spinal cord-injury model [178]. In clinical trials, the intravenous infusion of autologous MSCs appears to be a feasible and safe therapy that may improve functional recovery in patients with severe cerebral infarcts [179]. As the microenvironments appear important in stem cell lineage specification, MSCs are shown to specify lineage and commit to phenotypes with extreme sensitivity to tissue-level elasticity, see Figure 6 [180]. In GG hydrogels, MSC displayed the best phenotype with spreading actin in 0.8% and 1.4% GG gels. In hybrid hydrogel, higher proliferation rate and better phenotype were detected in a GG/KGM=0.6/1.0 gel (stiffness 60Pa). In all gels, there are no clusters detected. It may indicate that the pore size

of these gels is suitable for MSCs migration and stiffness of MSC may prefer 100-200 Pa. For the further study, neuron markers can be used to check the cultured MSC to see if there is any special trend in cell development. To enhance cell survival and proliferation, the modified gel with adhesion molecule or peptide need to be considered.

Meningeal Fibroblasts was isolated directly from the biopsy of adult brain. They have been shown to be present in the acute phase in the CNS injury, as they promptly infiltrate the lesion site and form a glial-fibroblast interface that produces new basal lamina which is important to reforms the glia limitans [181]. In the cell culture result, the best dense network (C1Figure 33) formed by MF is seen in the 1.4% GG gel (stiffness 250Pa). In other GG gels and hybrid gels, MF cells are in more rounded shape or with slightly spreading of phenotype. Better proliferation appeared in hybrid gel of GG/KGM=0.6/1.0 (Figure 35) but less cell spreading compare to 1.4%GG gel. Figure 36 showed that MF can escape from the gel and form a monolayer on the surface of the well. This result may suggest that the MF prefers stiffer substrate since the edge of gel is softer than surface of the plate. It is in compliance with that the cells sense the environment and this will influence their cytoskeleton. Since MF was found in glia scar, it may indicate that glia scar has higher stiffness than ECM in other location of the brain. This can be the cue for the cells which will be used for glia scar treatment. This also gives a hint that stiffness designing needs to consider the location from where cells derived. In further study, MFs can be culture in 1.6%GG gel comparing to softer gels.

The cell proliferation rate was not determined in present work since the confocal scan can only provide estimated cell number. Therefore, it not possible to compare the difference of proliferation rate between 2D and 3D cell culture. This work will be done in future to find a solution to dissolve the gels and to obtain stained cells for cell counting, e.g. when trypon blue could be used to stain. The substrate of cell culture between 2D and 3D in present work are not same, another additional investigation can be done in future is to use each gel conditions coating a 2D control, respectively. This may help to see hints of adhesion possibility of the gels. Nutrient and oxygen supply is important in cell culture; the oxygen generator through nanofibres which can be constructed in cell culture gel may help the cell growth. Adding other growth factors like insulin [182] may promote NSC survival. Endotoxin test also need to be done in future cell culture.

6 Conclusion

3D hydrogel cell culture system is easy to say but difficult to do. Cell culture requires the materials which at least need following properties, biocompatible and/or biodegradable, mild gelation process, optimal stiffness, suitable pore size for both cell and supporting factors, or easy modification for adhesion peptide and factors. To design the hydrogel culture systems, the stiffness is the first concern since it fates the cell viability and adhesion as well as proliferation. The concentration of polymers, the presence or absence of ions (the used medium) and ratio of gel to cell suspension have the big influence on the stiffness. Different cells favour different stiffness due to the mechanosens in natural environment. The results of this study showed that the GG gels and the hybrid GG/KGM gels can support NSCs growth, 1.4% GG and 1.1% GG showed preferable characteristics for NSCs and the live/dead stain supported that 1.4% (stiffness 250Pa) is the best for NSCs viability. In the GG/KGM hybrid gels, the series based on constant 1.1%GG with various KGM revealed that NSCs proliferated well in these hybrid gels and formed neurospheres. The 1.4%GG based hybrid series showed less cells than in the 1.4% GG alone gel. From the cell culture result it can be seen that different cells prefer different composition and concentration. MSC cells showed the best phenotype and proliferation in 0.8% GG gel (stiffness 130Pa), where cells proliferated, migrated and spread evenly and formed a 3D structure. Meningeal fibroblasts culture confided that MF preferred the stiffer gel (1.4%GG with stiffness 250Pa) where MF formed a dense network which was not the case in any other gel. To improve these 3D cell culture systems for NSC adhesion and growth, it requires more investigations on the peptide modified hydrogels, new growth factor, co-culture with natural neighboring cells of NSCs, oxygen and nutrient supply, live/dead stain and cell counting.

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