The first symposium on bioprinting in tissue engineering

Preparation & Characterization of bioink & biopaper for Production of 3D Cell-Scaffold Hybrid Structures by Bioprinting Technique

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The Objective of This Study

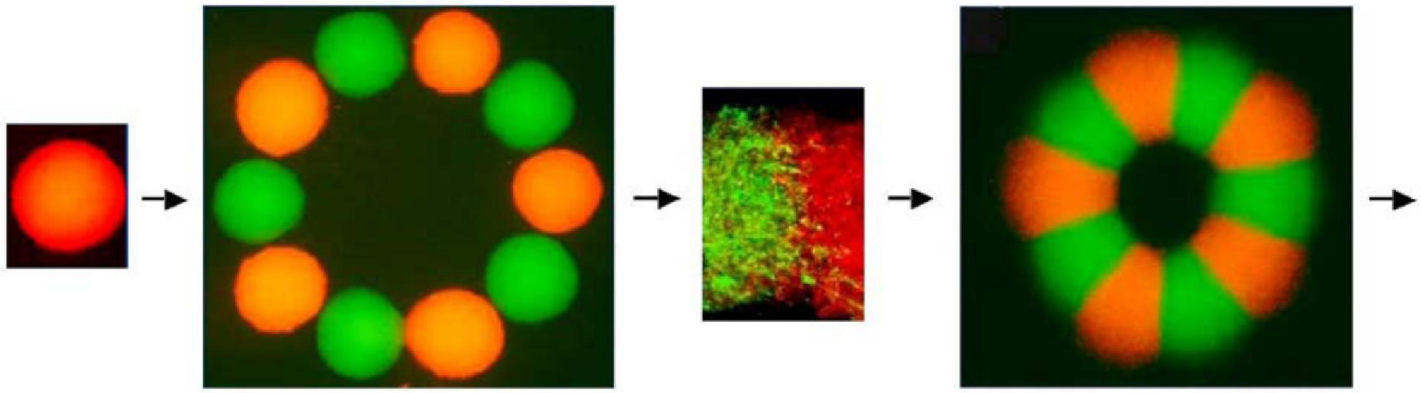
- ☑ ***First step****: preparing cellular aggregate as bioink*
- ☑ ***Second step****: preparation and characterization of a hydrogel substrate as a biopaper*
- ☑ ***Third step****: evaluation tissue fusion ability of optimized prepared bioink & biopaper*

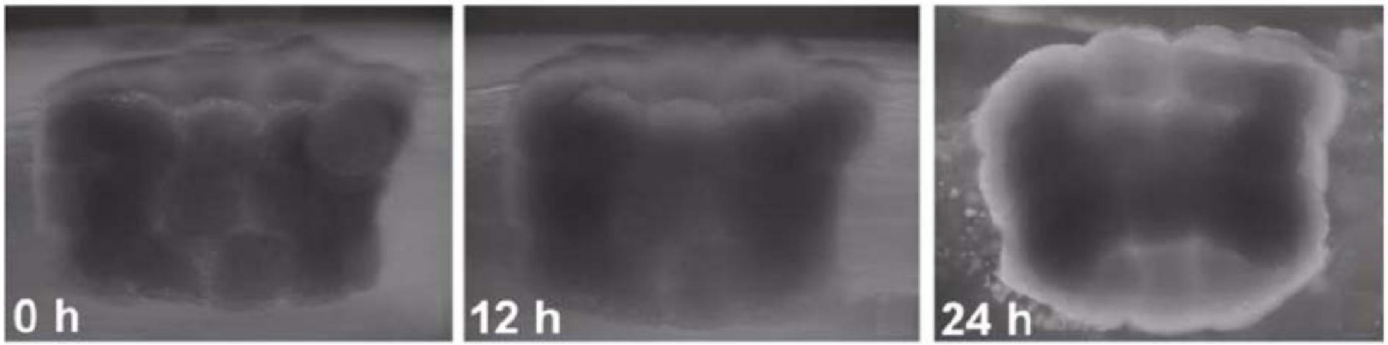
Preparing bioink: cell aggregates

Why Aggregate?

- ☑ *Mimicking native micro tissue structure and function*
- ☑ *Providing pre-builted small tissue blocks*
- ☑ *Containing many thousands of cells*
- ☑ *providing critical cell density*
- ☑ *Fusing immediately into 3D structures*
- ☑ *Saving time during organ maturation*
- ☑ *More survival experimental manipulations*

3D Fusion of Aggregates





… a branching tube by lateral deposition of bioink particles or …

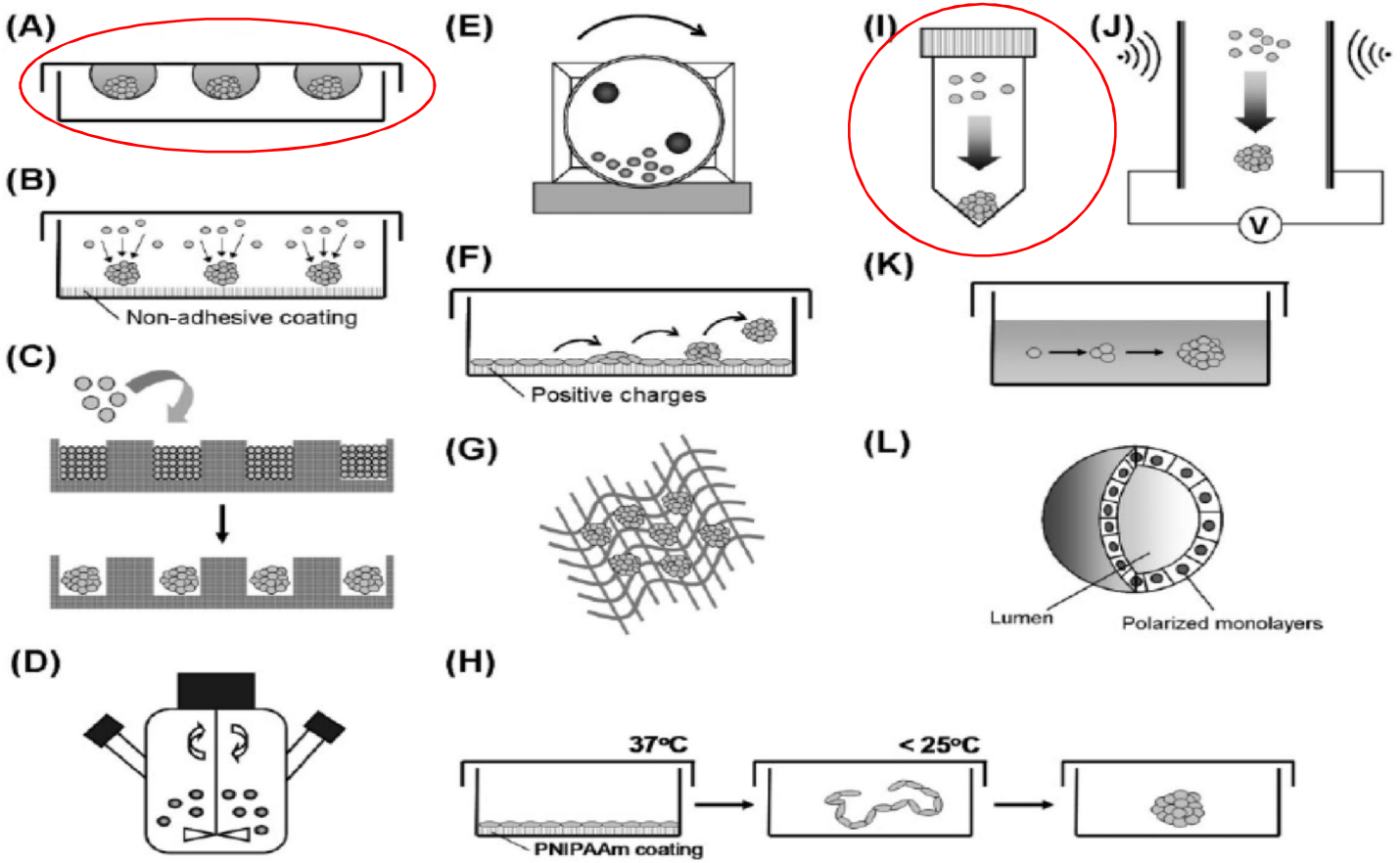
3D Cell Culture Method

- *Native tissues are three-dimensional*
- *It is a well-established fact that cells show different biological activity in 2-D and 3-D environments.*
- *Culturing cells in a 3D context produces distinct cellular morphology and signaling events compared with a rigid two-dimensional (2D) culture system.*
- *Cellular aggregate production needs 3D culture method.*

An Ideal Method

- *First : be a scalable method.*
- *Second : produce homogeneous aggregates in size*
- *Third : don't induce significant cell injury*
- *Fourth, don' compromise the cells capacity for sequential tissue fusion.*
- *Fifth : be easy , available and economic*

Different 3D Culture



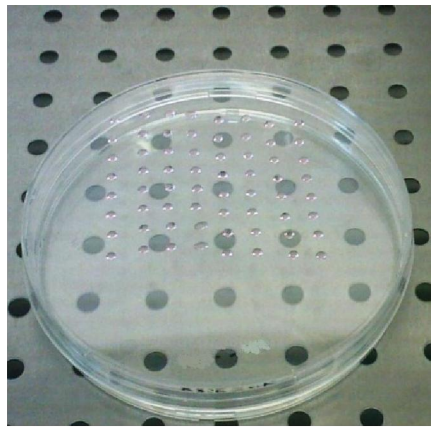
(A) Hanging-drop culture. (B) Single cell culture on nonadhesive surface. (C) Micromolding techniques. (D) Spinner flask culture. (E) Rotary cell culture systems. (F) Hepatocyte self assembly on Primaria dishes. (G) Porous 3-D scaffolds. (H) The use of PNIPAAmbased cell sheets. (I) Centrifugation pellet culture. (J) Electric, magnetic or acoustic force cell aggregation enhancement. (K) Monoclonal growth of tumor spheroids. (L) Polarized epithelial cysts.

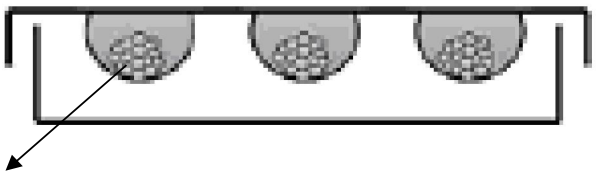
3D Cell Culture

Chinese hamster ovary cell (CHO) were cultured in RPMI 1640 cell culture medium containing 10% fetal bovine serum 1% Penicillin and Streptomycin.

Hanging Drop (HD)

- ***Hanging drop*** *culture is a widely used embryonic body (EB) formation induction method.*



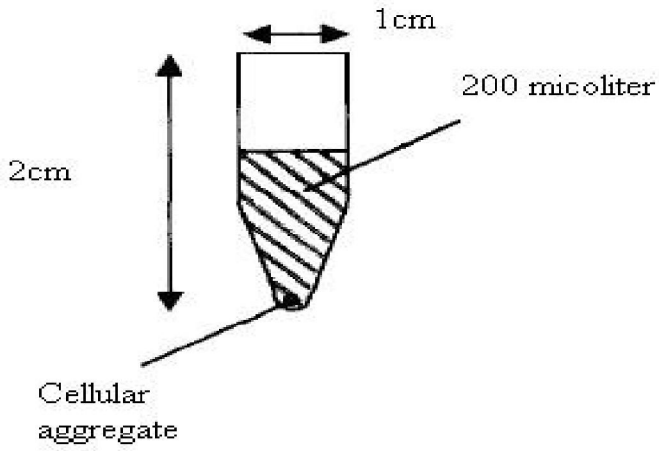


Each drop: 20-µL

- *We prepared 20-µL drops containing approximately 5000, 10000, 25000, 50000 on the inner side of the lid of a 15 cm diameter tissue culture Petri dish .*
- *Samples were named : HD5, HD10, HD25, HD50respectively.*

Conical Tube (CT)

- *The culture for aggregate was performed in a polypropylene 200µL conical microtube of round bottom that is, the conical tube (CT) method .*



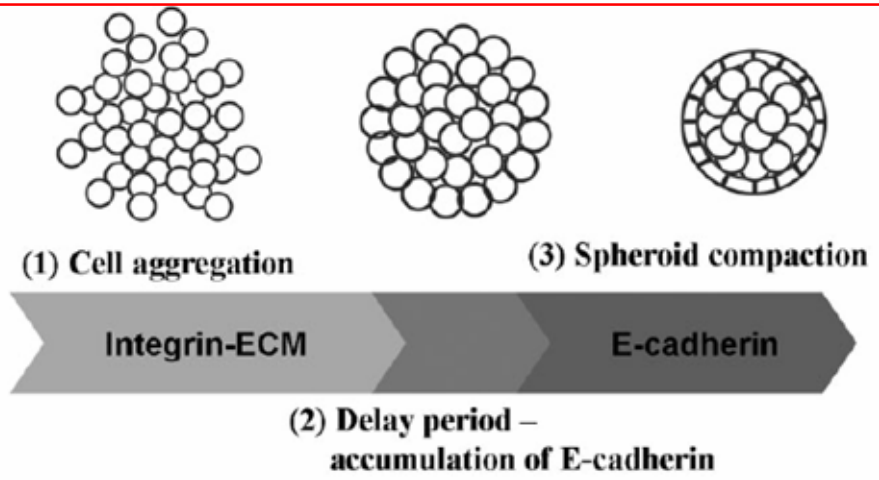
- *200 µL of cell suspension containing 5000, 10000, 25000, 50000 cells were placed in the microtubes then was centrifuged at 2000 rpm for 5 minutes*
- *Samples were named : CT5, CT10, CT25, CT50 respectively*

Pre-Culture Period

Aggregate Formation

- *Aggregate formation is inherently a three step process .*
- *Any method that concentrates suspended cells to high density can potentially facilitate aggregate formation.*

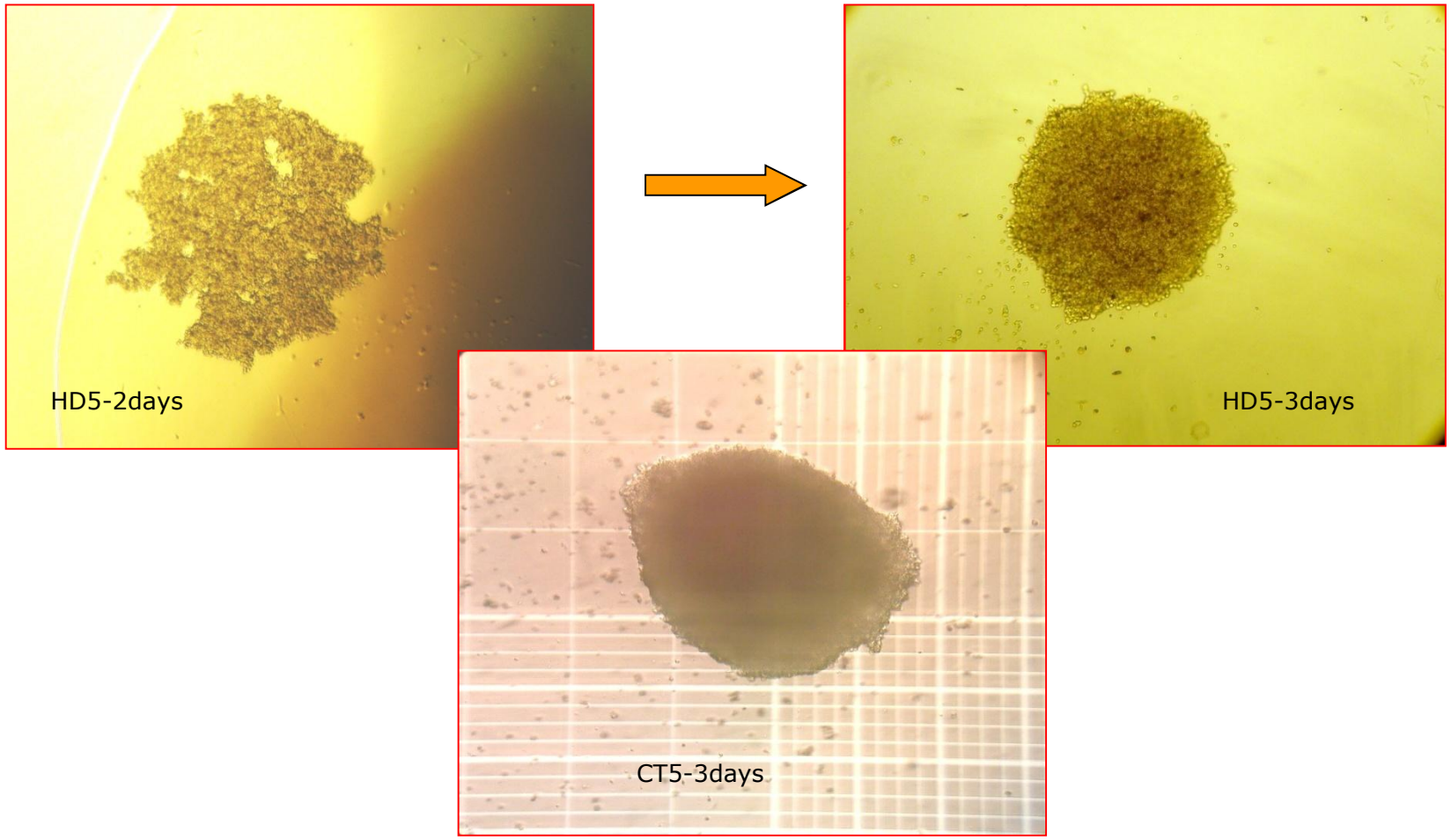
In comparison to HD that cells sediment freely by gravity force, centrifuged cells are forced into the aggregate configuration immediately



Size & Shape Analysis

- *The aggregates were observed by an Olympus phase contrast inverted light microscope equipped with a camera.*
- *captured images were analyzed by (Motic Image Proplus) software for determining altering of aggregate's radius by time.*

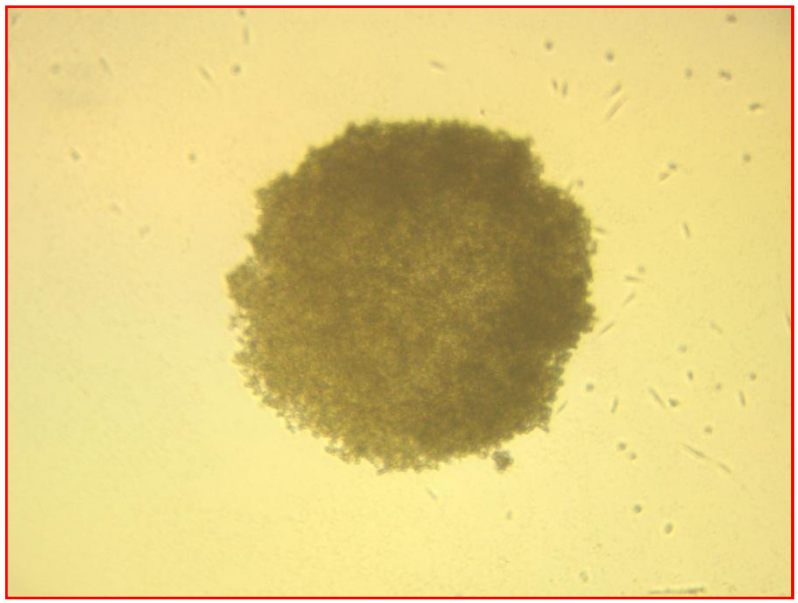
Aggregate Shape



The general shape of the CT aggregates was more irregular, rather than smooth.

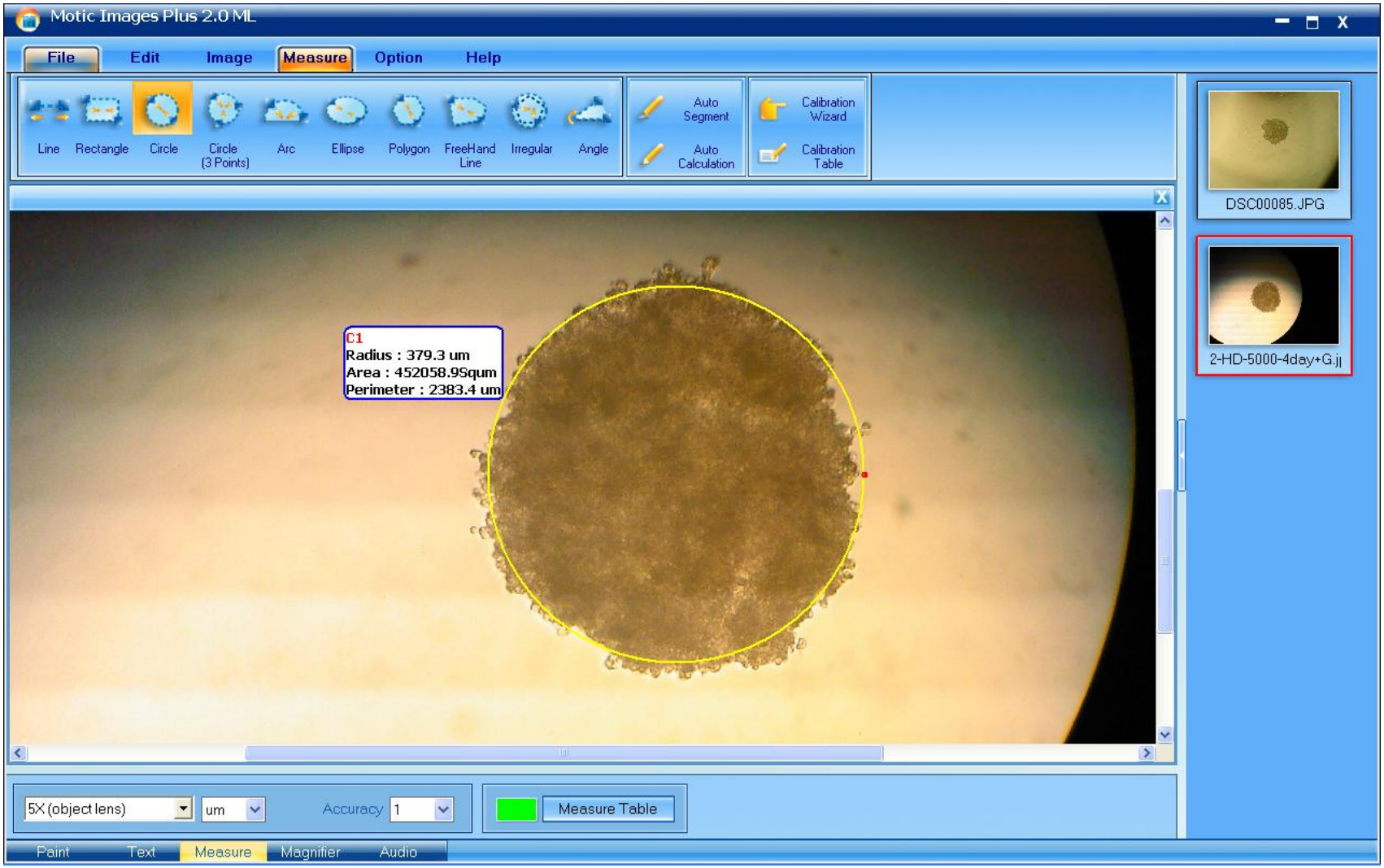
Aggregate Shape

HD5-3days



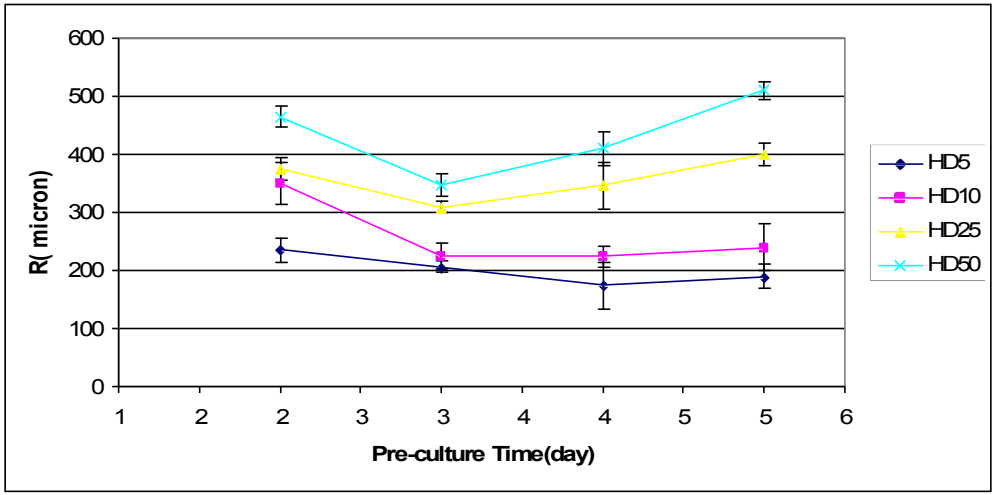
HD10-3days

Aggregate Size Measurement

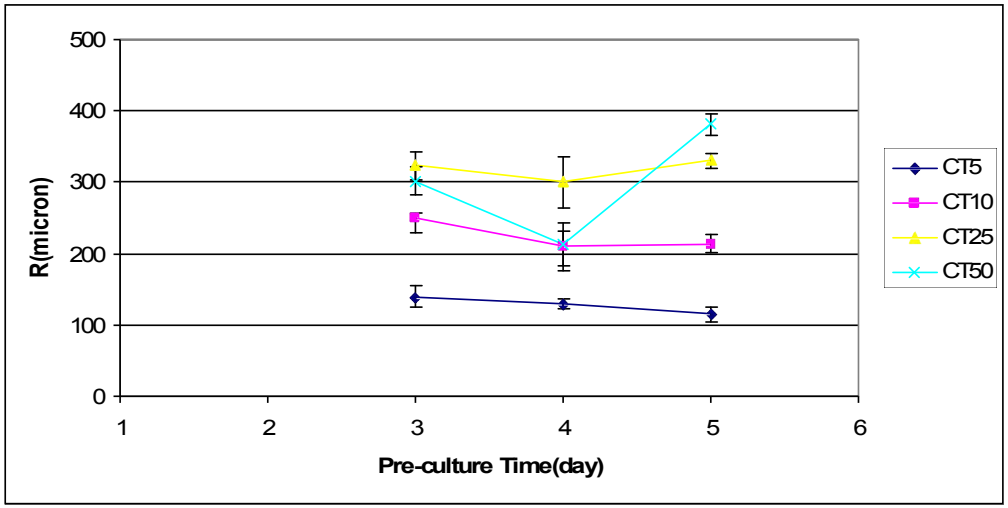


Aggregate Size

- *The minimum size of an aggregate during pre-culture was lower than 400 micron for HD samples and 300 for CT*

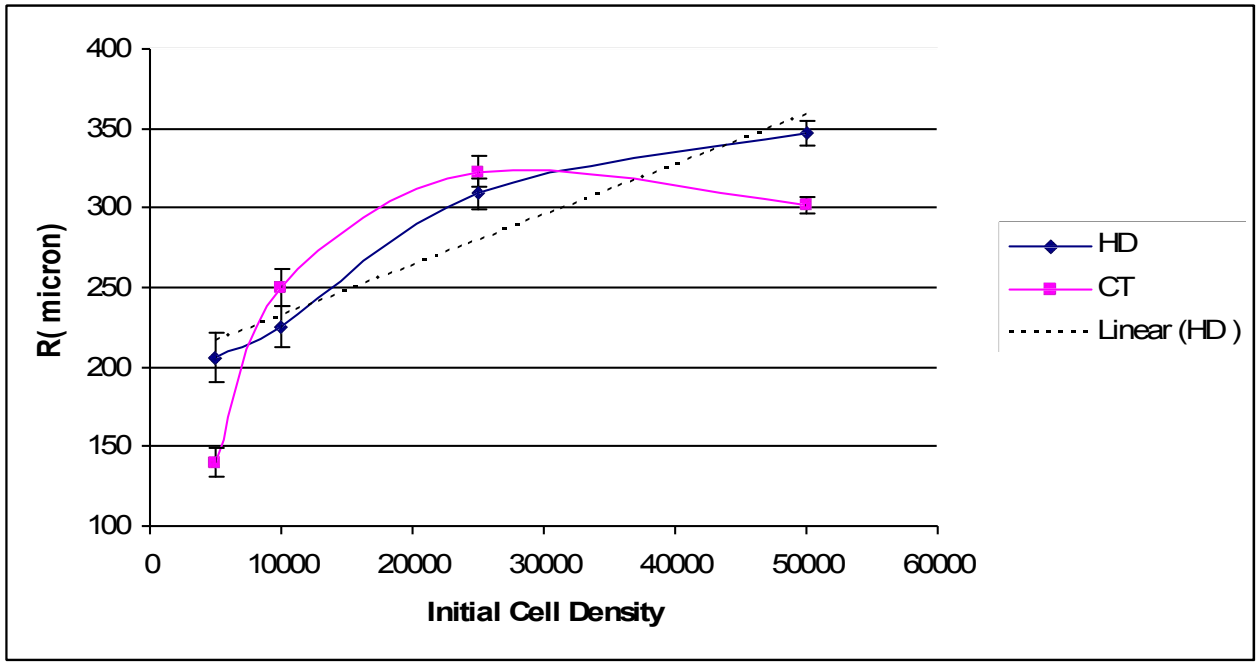


- *The CT aggregate in same initial density and pre-culture time is smaller than HD one.*

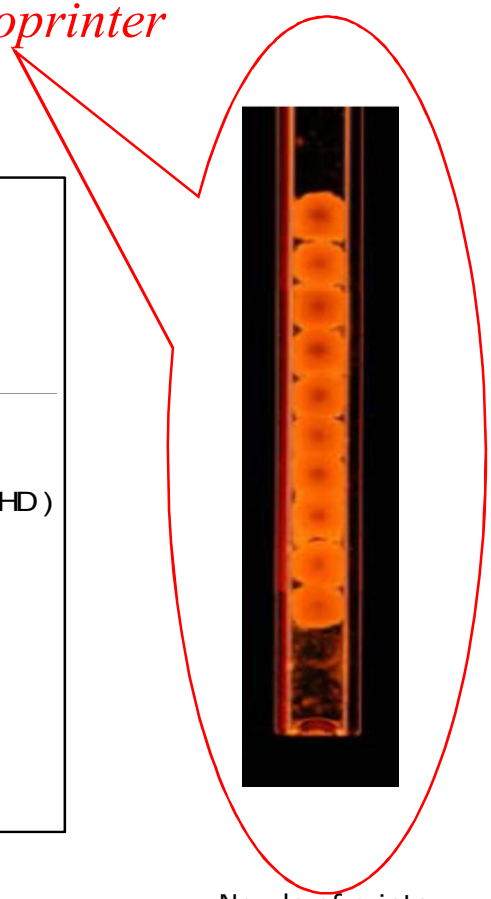


Size Controllability

- *Importance of size control*
  - *Cell viability: Diffusing of nutrient*
  - *Aggregate deposition by bioprinter*



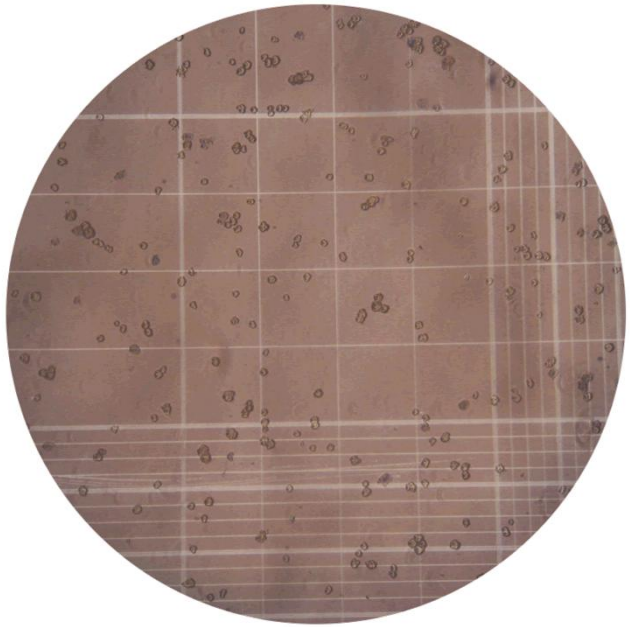
In third day of pre-culture



Nozzle of printer

Analysis of Cell Viability

Aggregate cell viability was determined by Trypan Blue exclusion tests after disruption into single cells.



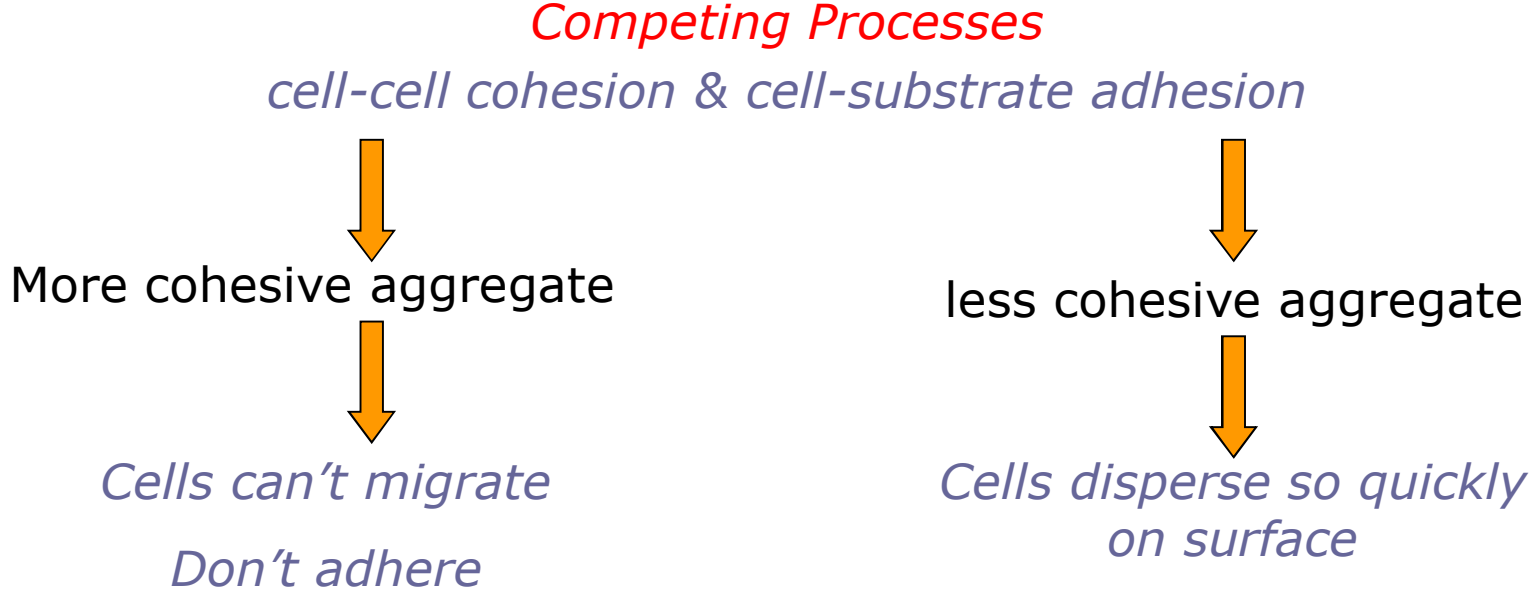
Viability

Average percent of aggregates viability during per-culture

	2day	3day	4day	5day
HD5	100	100	100	90
HD10	100	97	88	70
HD25	98	93	67	55
HD5	93	82	56	30
CT5	100	100	90	88
CT10	100	92	80	76
CT25	70	66	60	50
CT5	65	50	45	23

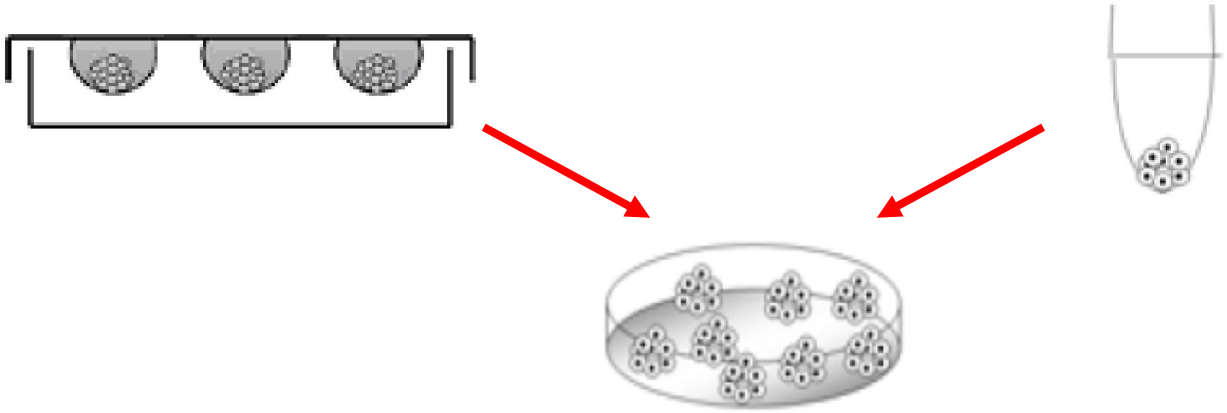
Tissue Spreading Assay

- *Tissue spreading over a substratum is a fundamental process in animal development, wound healing, and malignancy.*
- *The nature of interactions between cells and scaffolds on the cellular level at least initially is basically two-dimensional.*



Tissue Spreading Assay

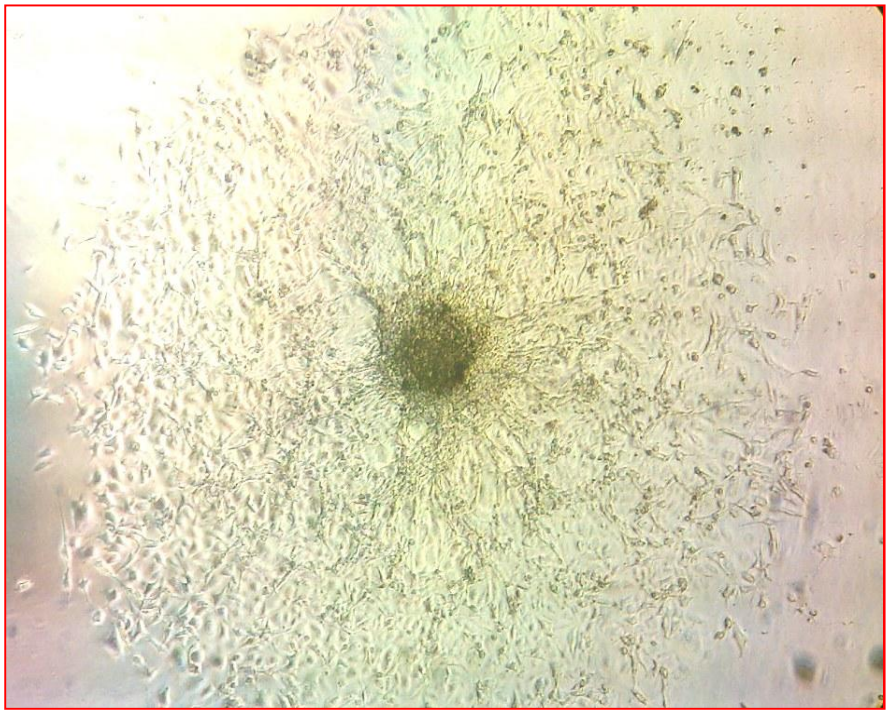
- *For estimation of Tissue spreading ability of obtained aggregate over a substratum and ability of interaction on 2D adhesive substrate, spreading aggregate cells on tissue culture plate was examined by microscopic observation*



- *tissue spreading on surface was evaluated by measuring of Expansion Parameter (Re/Ri).*

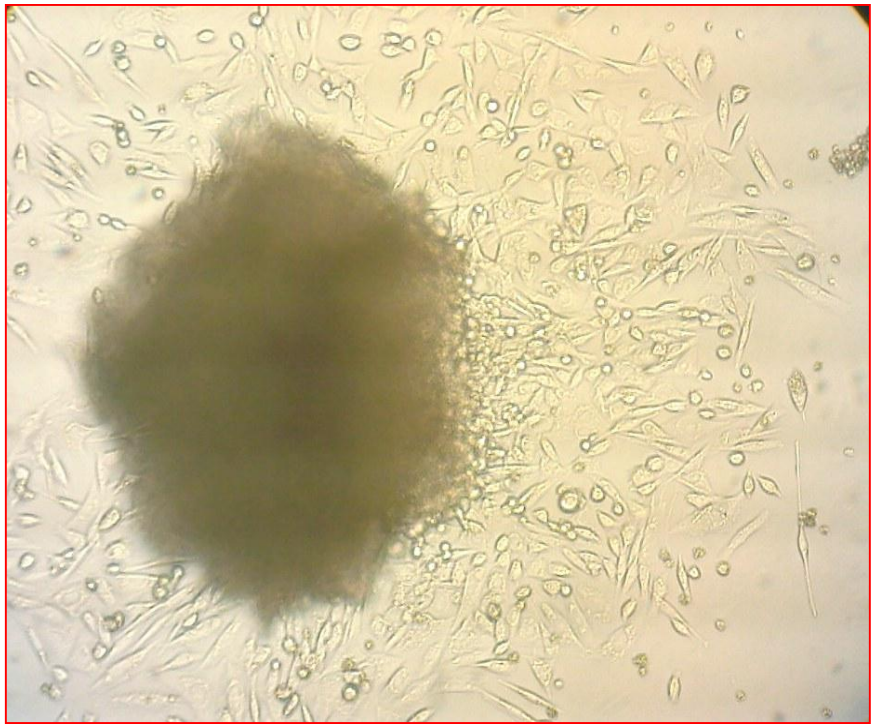
Re: expansion radius & Ri: initial radius

Cell Spreading

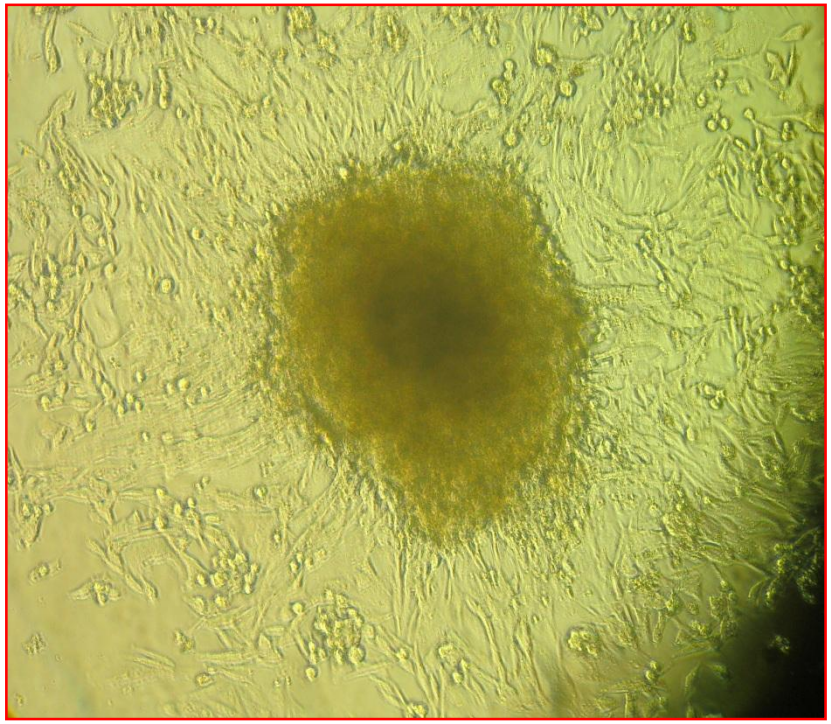


HD5-4day (×40)

HD25-3day (×100)

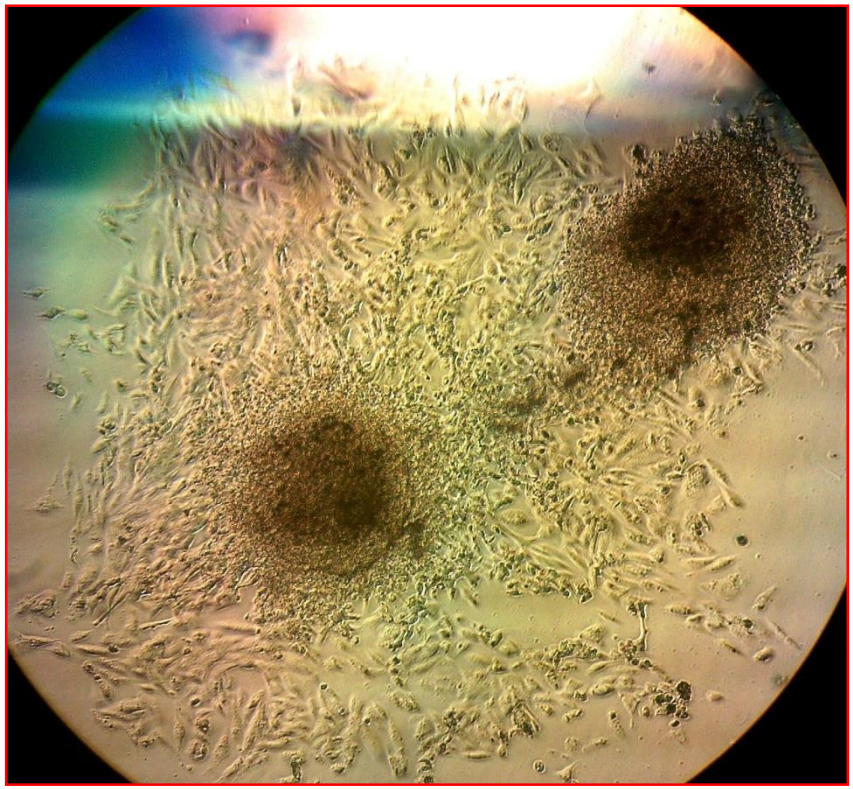


Cell Spreading

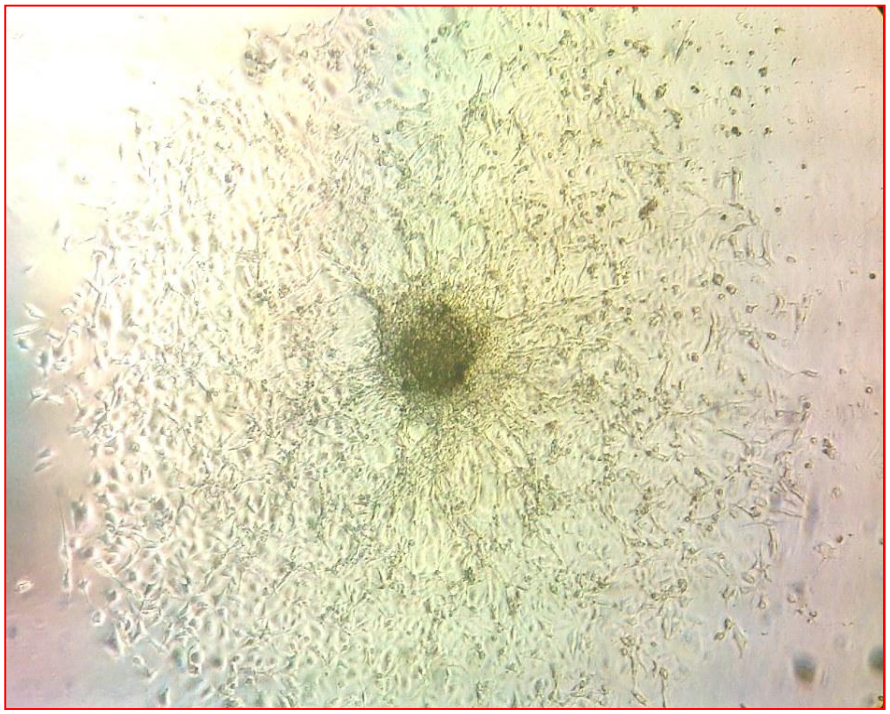


HD5-4day (×100)

HD5-4day (×40)



Estimated Expansion Parameter



Significant extension

	1day	4day
HD5	3.00	6.77
HD10	2.73	3.40
HD25	2.03	2.40
HD50	1.00	1.00
CT5	1.10	3.70
CT10	1.00	1.20
CT25	1.00	1.00
CT50	1.00	1.00

No extension

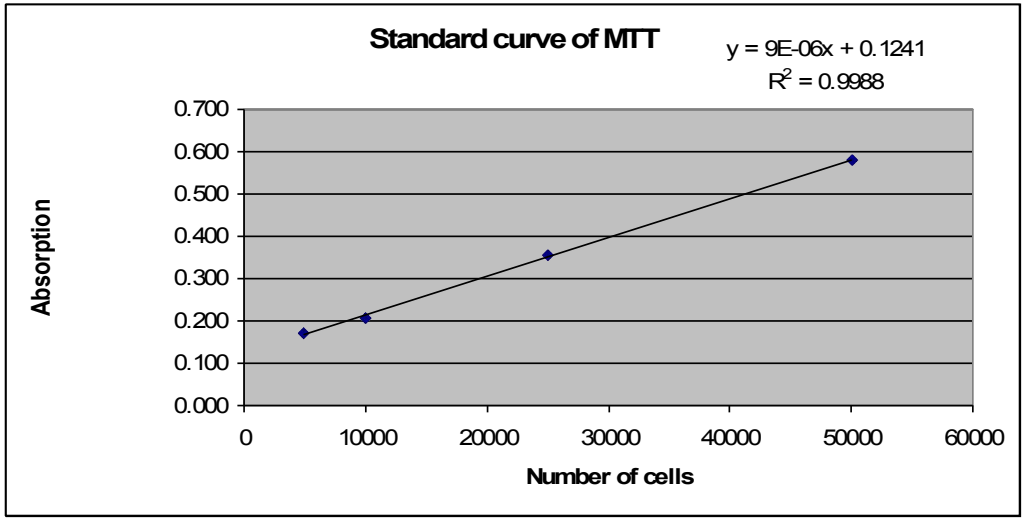
Evaluation of Proliferation Ability

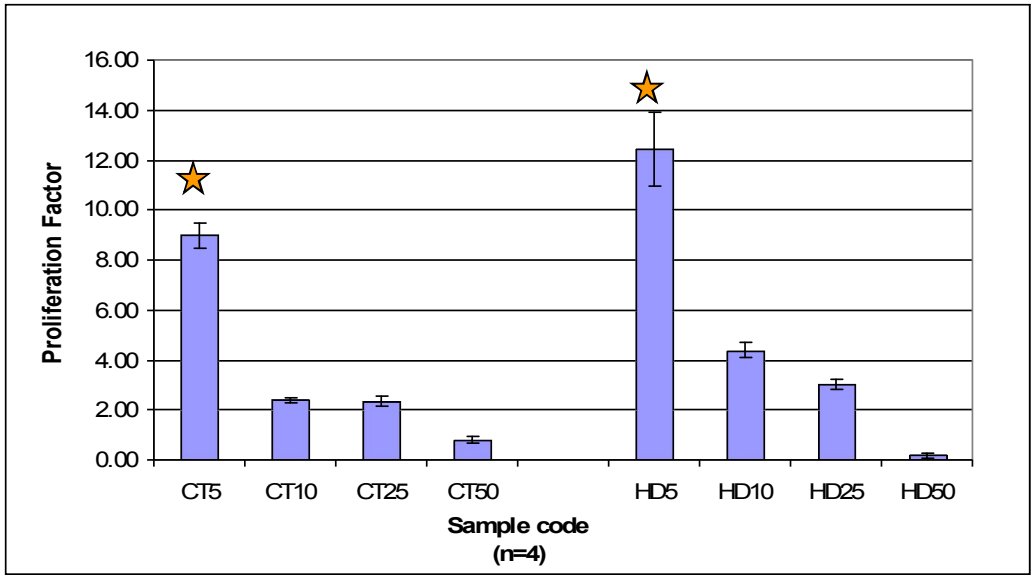
- *For investigation of cells proliferation and growth ability in the form of aggregate, aggregates went through MTT.*
- *MTT test was improved and modified for examining number of aggregates cells and aggregate's cell proliferation over culture time.*
- *Data estimated final number of cells in each well containing aggregate after 3 day.*

$$\text{Proliferation factor} = \frac{\text{approximate final number of cell}}{\text{initial load cell per drop/tube}}$$

Cell Proliferation

- *CT5 & HD5 considerably multiplied by 9 and 12.44 factor respectively.*
- *It is represented embossed proliferation ability of these aggregates.*
- *Obtained value for CT50 & HD50 are less than 1.*





Conclusion: Part1

- *Based on obtained date, minimum size of obtained aggregates are in the appropriate range indicated by other studies.*
- *Hanging drop method provides better size controllably*
- *CT aggregate can be retrieved easier.*
- *In comparison to HD, at the same time and initial cell density, CT aggregates are smaller but less viable.*
- *CT technique results more cohesive aggregate but HD ones have remarkable interaction to substrate and proliferate fast.*

By considering all criteria, Hanging Drop is able to produce aggregate with desirable characteristic. Aggregates produced by this method in low density, 5000 and 10000, are favorable for printing application.

Preparation Biopaper

Hydrogel as a Biopaper

- *Hydrogels are the only biomaterial can be used as a biopaper.*

Characteristics of ideal hydrogel for organ printing.
• Bioprocessible (dispensible and fast solidification)
• Biomimetic (functional arginine-glycine-aspartic acid peptides for improving viability)
• Biocompatible (nontoxic, high cell viability)
• Intelligent (stimuli-sensitive)
• Tissue fusion permissive (optimal physicochemical properties)
• Shape maintanence (preventing construct melting and distortion)
• Hydrophylic (efficient diffusion)
• Biodegradable (removable on demand)
• Naturally derived hydrogels (collagen, fibrin, hyaluronan based)
• Pro-angiogenic and loaded with survival and angiogenic factors (enhancing bioprinted construct viability)
• Affordable (relatively low cost)
• FDA approvable (noncancerogenic and nonimmunogenic)

Material Selection

Temperature sensitive hydrogel *can be best candidate for biopaper applications*

Type	*Hydrogel*	*Origin*	*Properties*
Thermosensitive	Agarose	N	low cost; dubious biodegradability; low physical qualities; resistant to protein adsorption
	Collagen	N	Adhesive extracellular matrix component; shrinkage batch variance; coll II > I for chondrogenesis
	Gelatin	N	weak at physiological temp; limited to combined use/chemical crosslinking
	Matrigel	S	commercially available; expensive
	PNiPAAm	S	non-degradable; activation platelets?
	Pluronics	S	fast dissolution; induction of hyperllpidemia in rats; inverse thermosensitive
	PEG triblocks	S	FDA approved; inverse thermosensitive
	Poly(propylene fumarate-co-ethylene glycol)	S	
	Chitosan	N	structurally similar to GAG; intrinsically antibacterial

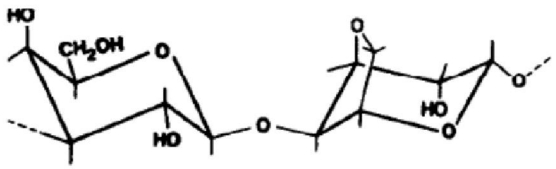
Blend Hydrogel

Blending is a simple method to combine the advantages of different polymers. The resulting polymer blends may show synergistic properties.

Agarose:

a plant polysaccharide present in the cell wall in some algae

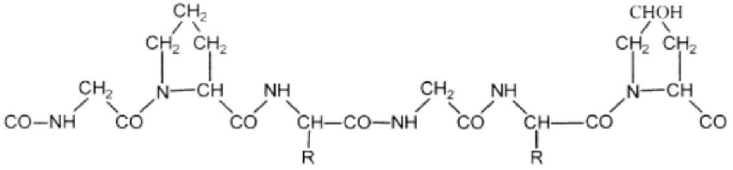
- ☑ *Thermoreversible hydrogel*
- ☑ *Soft tissue-like stable mechanical properties*
- ☑ *Biocompatible (bioinert)*
- ☑ *Slow biodegradation*
- ☑ *Low price*
- ☒ *Significant low cell adhesiveness and cell proliferation*



Gelatin:

a protein derived from the partial hydrolysis of collagen

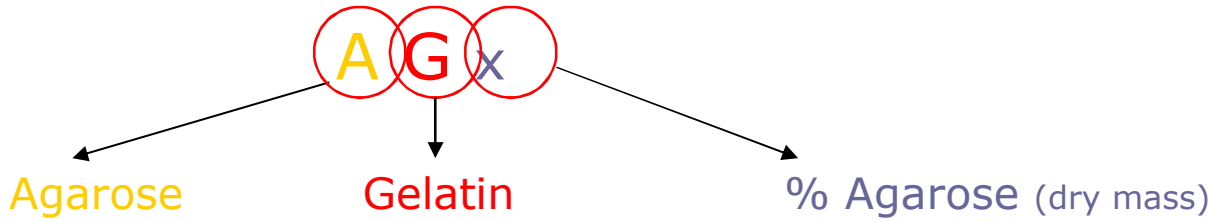
- ☑ *Thermoreversible hydrogel*
- ☑ *Biocompatibility (bioactive)*
- ☑ *Excellent cell adhesion*
- ☑ *Low price*
- ☒ *poor mechanical properties & instability under physiological condition*



Compatible Components: Hydrogen Bond, Electrostatic Interaction

Sample Preparation

- *The hydrogels used in this study were prepared by blending of gelatin, agarose.*
- *The blend hydrogel were prepared by taking agar and gelatin in different ratio and dissolving them in hot deionized water (gelatin: 70°C, agar: 90°C).for making 3% homogenous solutions.*
- *Solution was kept in room temperature till gel formation then transferred into 4°C.*



Sample code: AG100, AG75, AG50, AG25

Sample classification

Sample classification and compositions of hydrogels

Sample code	Gelatin (g)	Agarose (g)	Double distilled water (ml)
AG100	0.0000	0.0300	10.000
AG75	0.0075	0.0225	10.000
AG50	0.0150	0.0150	10.000
AG25	0.0225	0.0075	10.000

Gel Point Determination

Gel point:

Sol to Gel transition point

Network Formation

↓

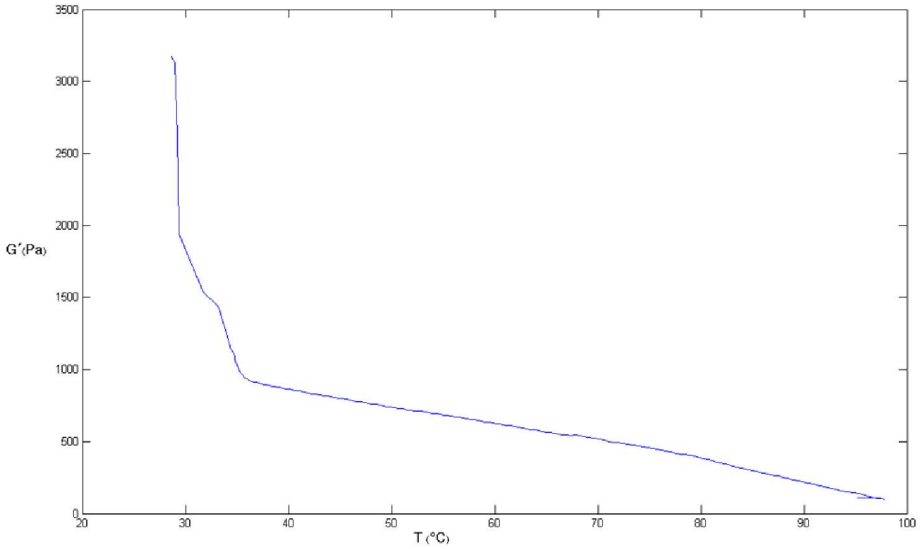
Viscose ⟷ Elastic

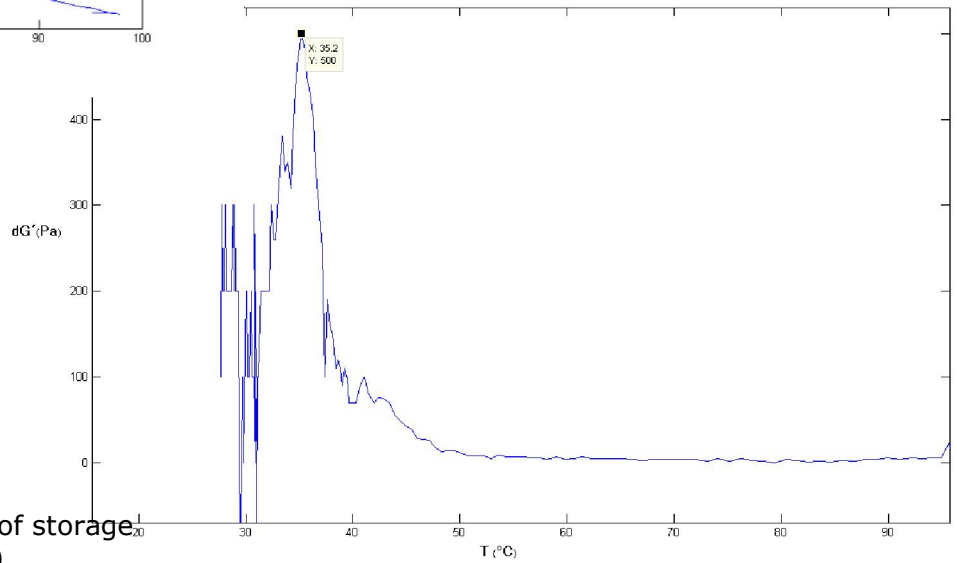
↓

Rheological study

- *The rheological experiments: a plate–plate dynamic rheometer using equipped with a peltier element for temperature adjustment.*
- *solution placed between the two heated (90°C) plates and covered with silicon oil to prevent drying.*
- *The oscillation experiment : deformation of 1% and an angular frequency of 1 Hz.*
- *Data (**G′ and η*)** were continuously recorded during the temperature sweep, which cooled from 90°C down to 25°C*

Gel Point Determination





Sample Curve of storage modulus (a) and first derivative of storage modulus (b) as a function of temperature (AG50).

Gel Point Determination

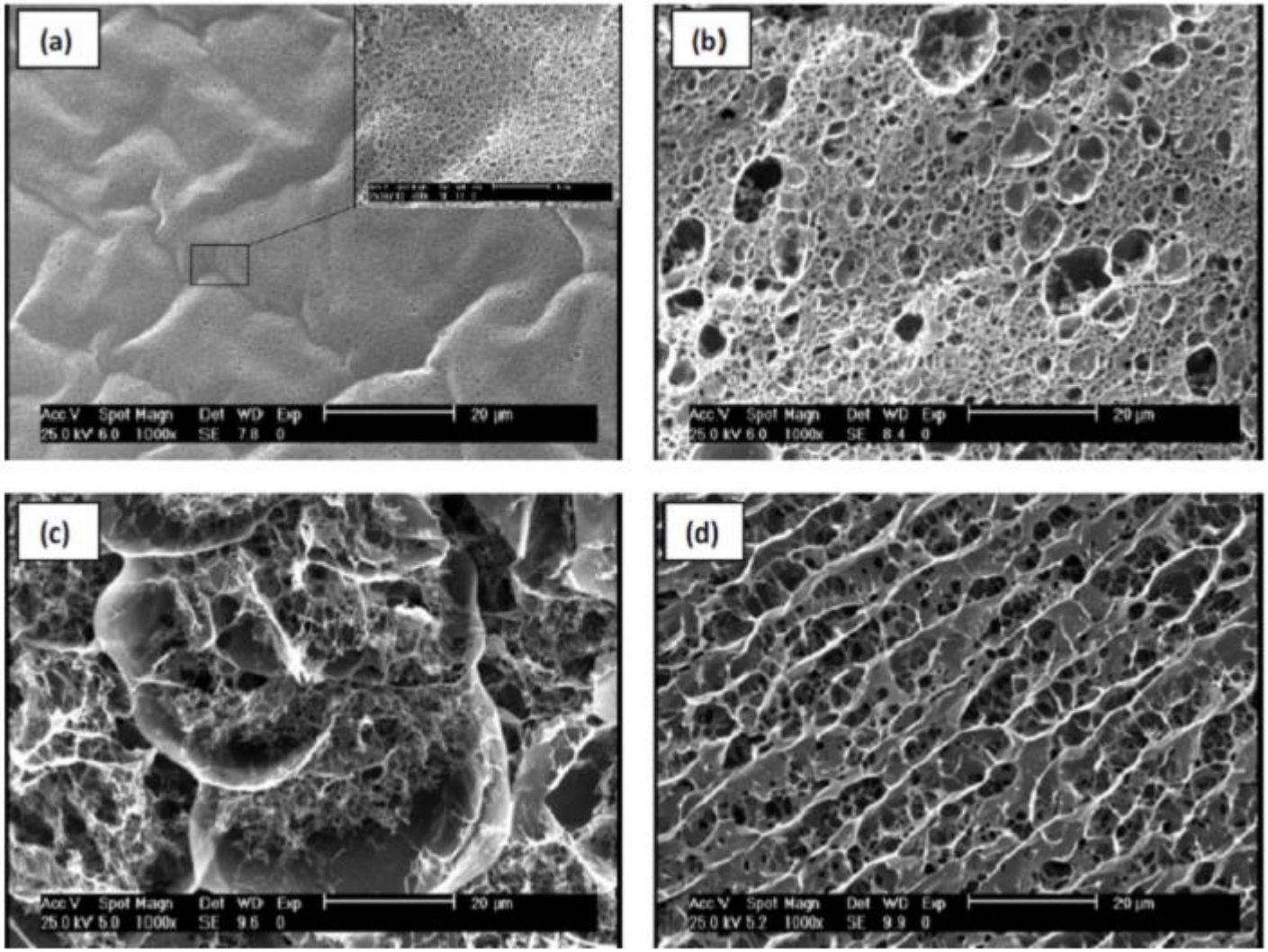
Gel point of blend hydrogels based on rheological study

Sample code	AG100	AG75	AG50	AG25
Gel point (°C)	39 ± 0.3	37.1 ± 0.8	35.2 ± 0.5	28 ± 1.5
Complex viscosity at gel point (Pa.s)	$1.25 \pm 0.1 \times 10^4$	$1.75 \pm 0.08 \times 10^2$	$1.63 \pm 0.1 \times 10^2$	$9.34 \pm 0.12 \times 10^2$

Acceptable

(37°C ± 2)

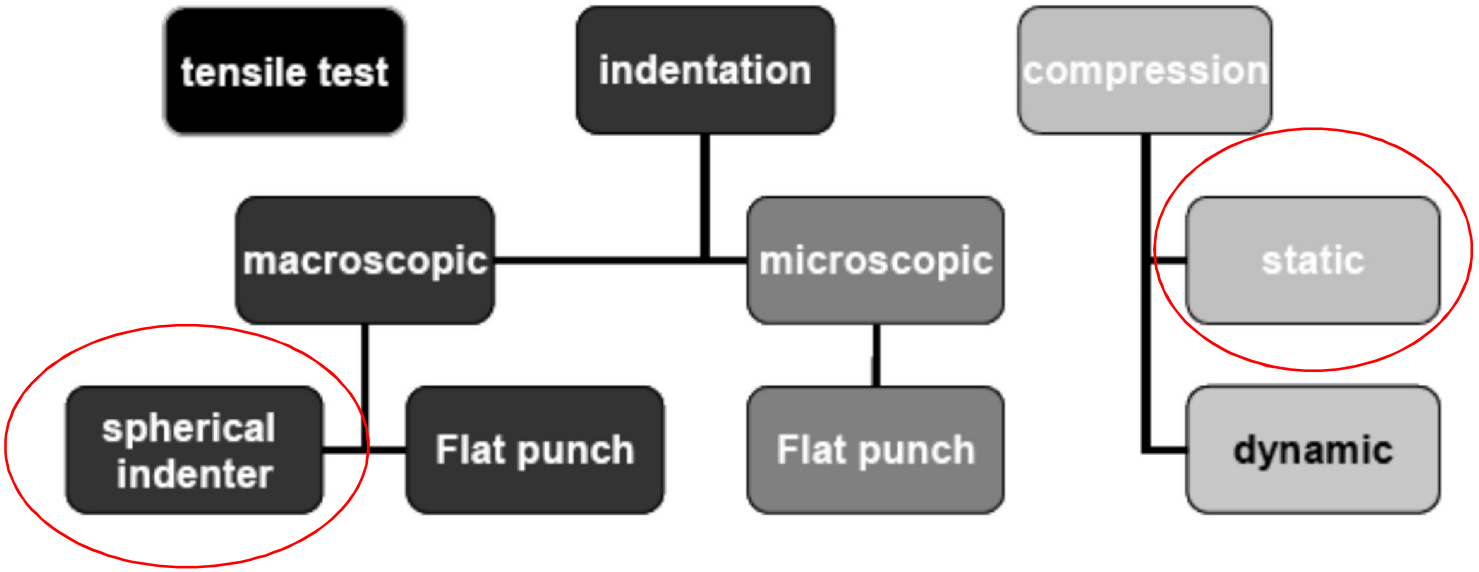
Study of hydrogel morphology



SEM micrograph of freeze dried hydrogels

Mechanical Properties & Stability

- *Characterizing the mechanical properties of gels can be troublesome because they are "soft solids".*



Mechanical Properties & Stability

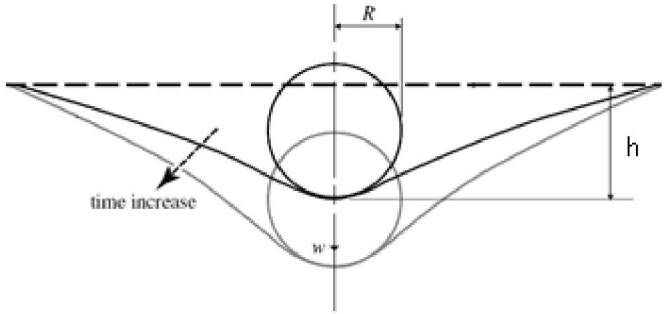
- *Uniaxial Compression Test: Estimation of Young modulus and Stiffness.*

- *The force required to compress the hydrogel and the amount of deformation are used to derive a stress versus strain graph from which the compressive modulus and compressive strength can be determined.*

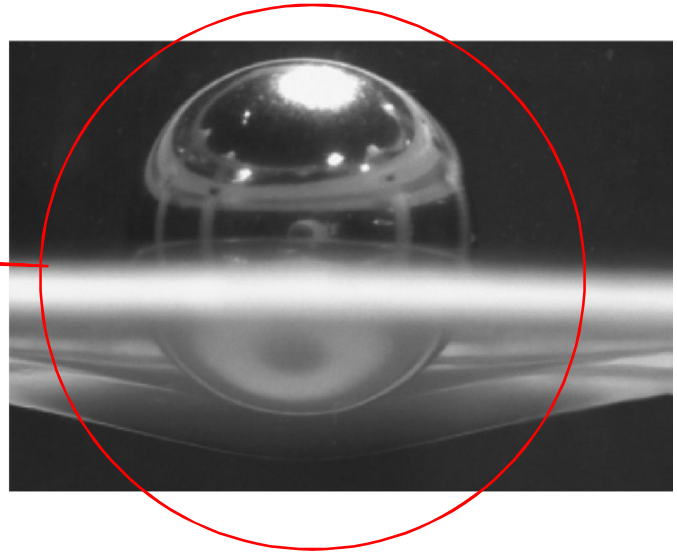
  $E = K \times (L/A)$

Mechanical Properties & Stability

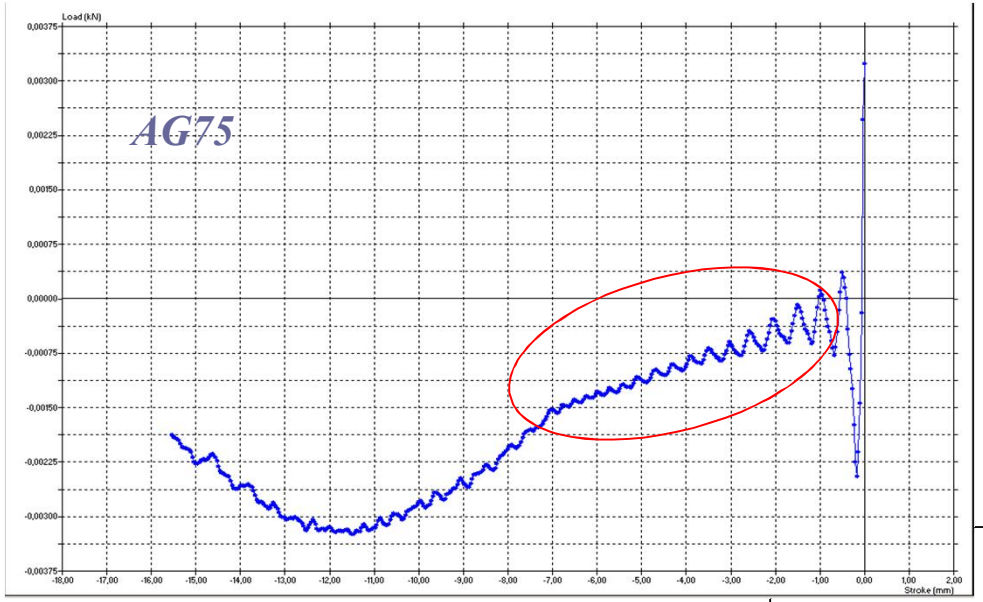
- *Indentation: a central indention of a disk of hydrogel using a ball of known weight and measurement of the corresponding displacement occurring at the centre.*



$$E = \frac{3 \times (1 - \gamma^2) \times F}{4 \times h^{1.5} \times r^{0.5}}$$

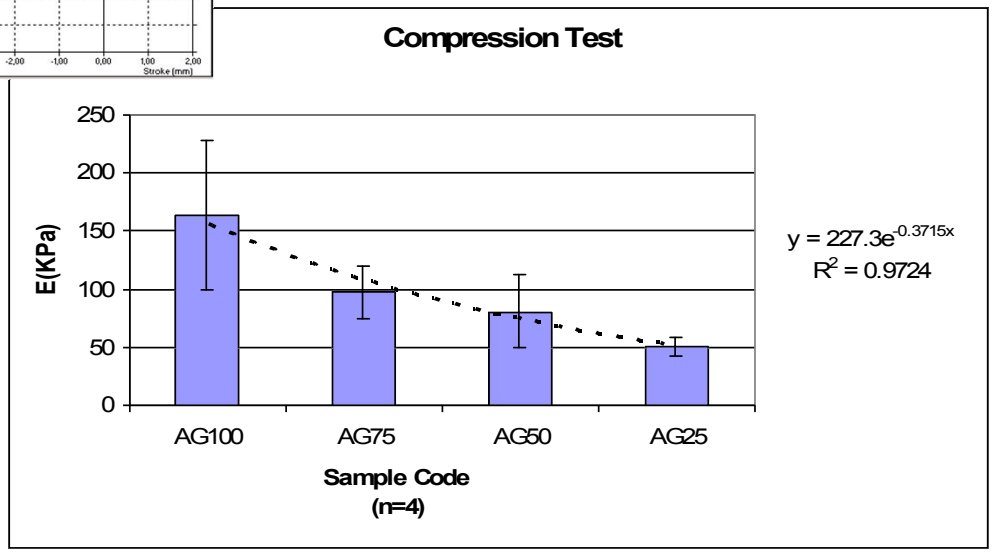


Mechanical Properties & Stability

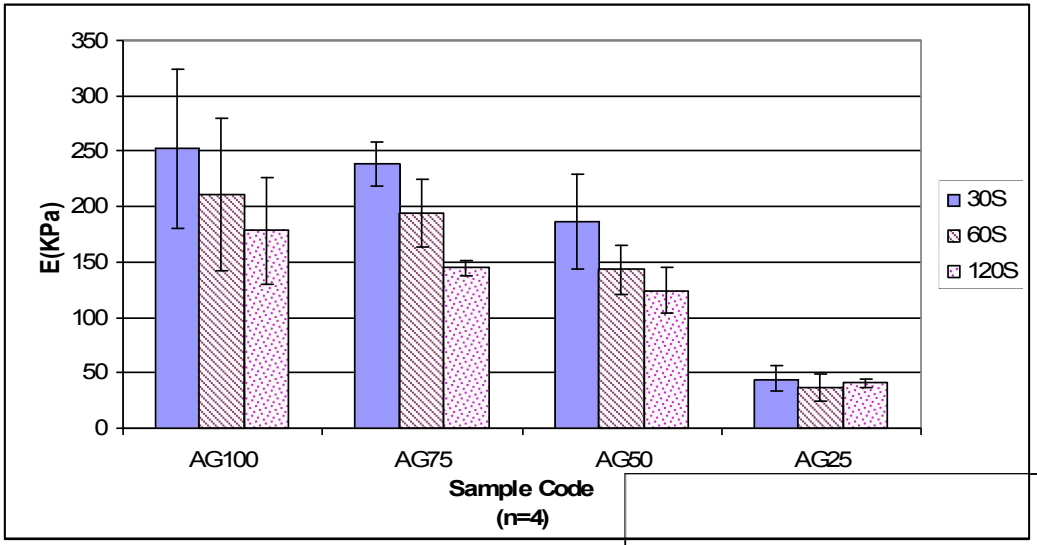


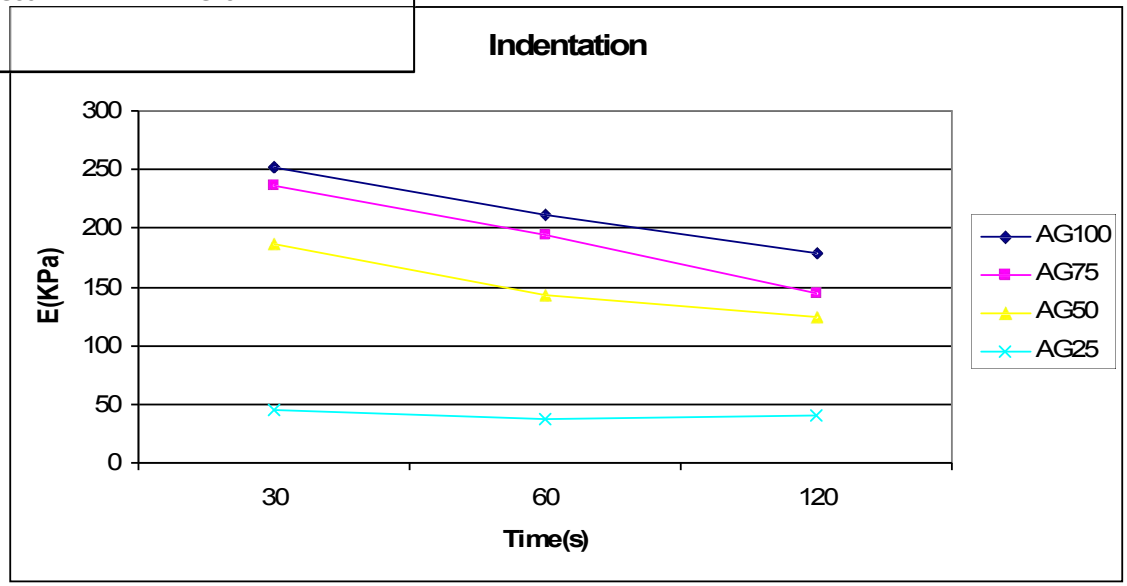
Slop indicates stiffness (k)

Curvature of the specimens decreases precision



Mechanical Properties & Stability





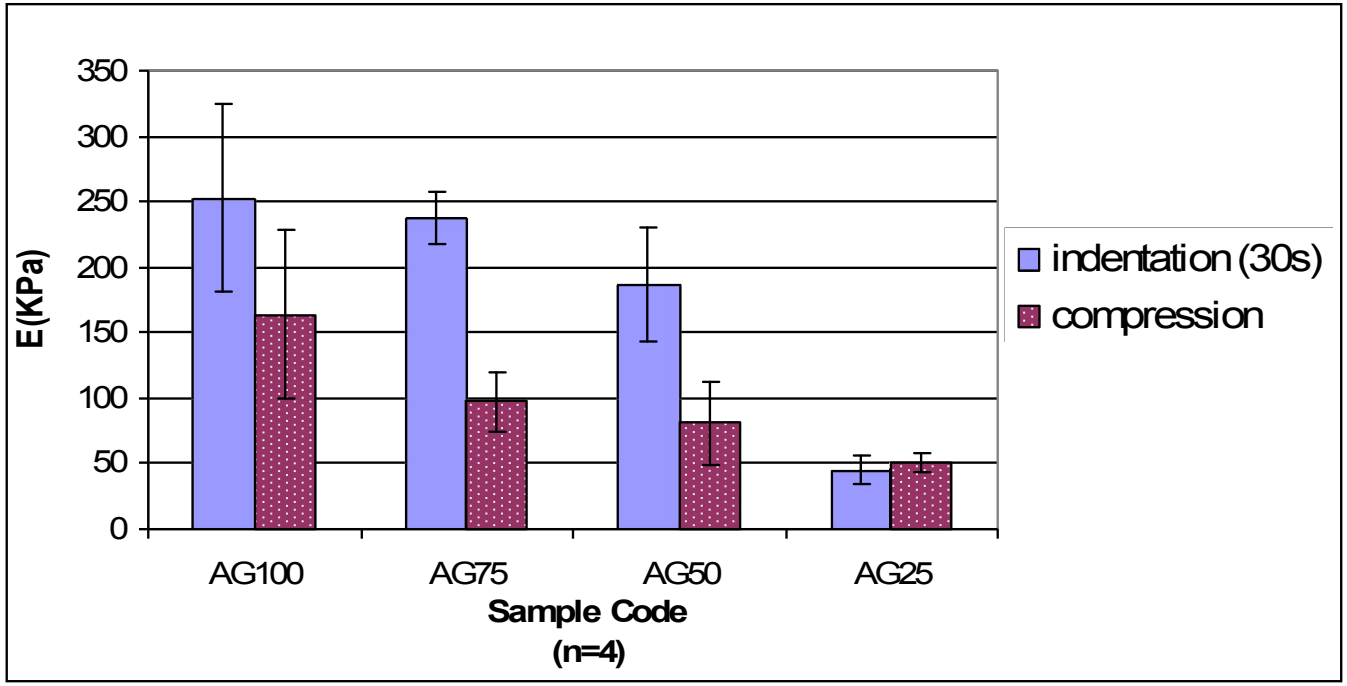
More Viscoelastic behaviors for:
AG100, AG75, AG50

More Elastic behavior for:

AG25

Mechanical Properties & Stability

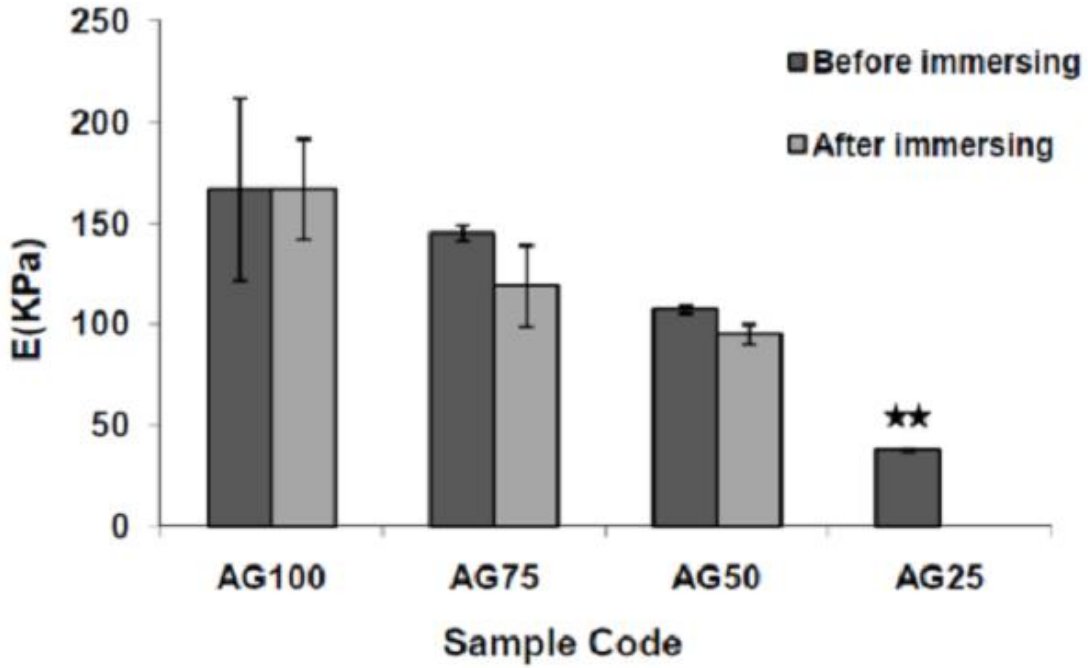
- *The compression measurements lead to low values for the initial modulus(about 100 KPa lower than indentation).*
- *This can be explained by the curvature of the specimens*
- *There was no differences for AG25*



Mechanical Stability

- *In physiological condition, most of hydrogel lose their mechanical stability.*

Sample code	*% Drop modulus*
AG100	*0%*
AG75	*18%*
AG50	*10%*
AG25	*rupture*



Biodegradation Analysis

- ☑ *% Degradation of dry mass (% Md)*
- ☑ *Rate of degradation*

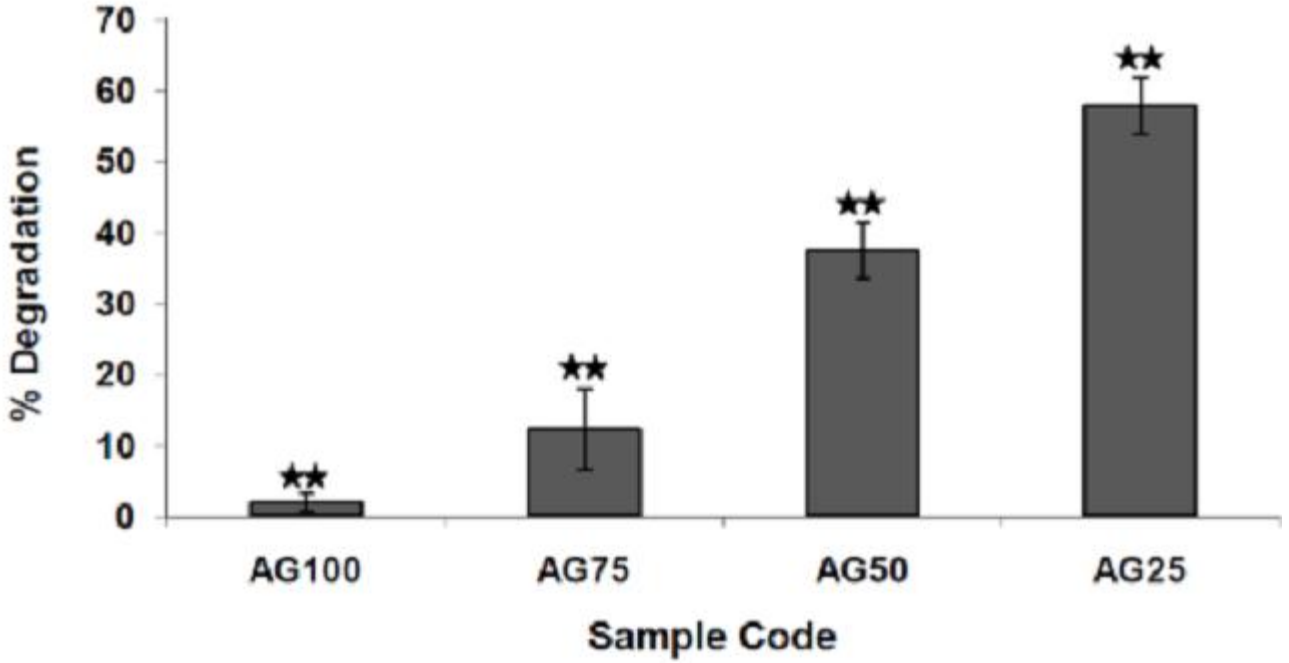
Sample (1cm diameter, 5mm thickness) immersed in 5 ml PBS at 37°C for maximum 7 days.

Degradation evaluated based on loosing of dry mass:

$$\%M_d = \frac{(W_0 \times 0.03 - W_1)}{(W_0 \times 0.03)}$$

Biodegradation Analysis

- *The ideal substrate should provide support in vivo until the cells are assembled and maturated enough to support themselves.*
- *The bio-ink droplets fuse and the bio-paper is eliminated by chemical physical or biological means.*



Integral Stability Analysis

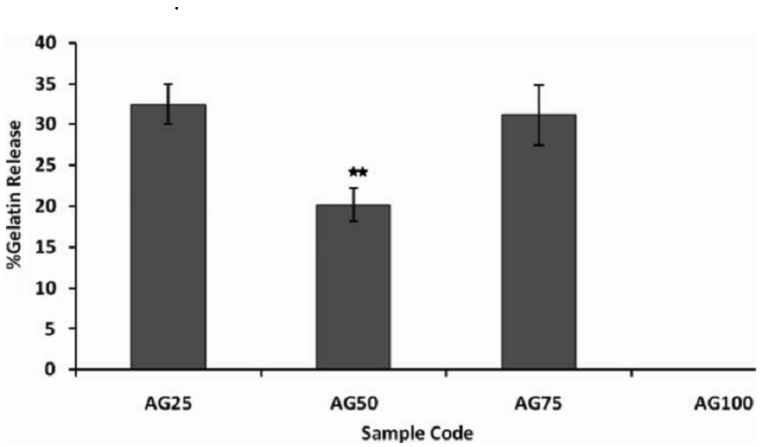
- *The integral stability was evaluated by studying in vitro release of gelatin molecules from the blend hydrogels.*
- *Concentration of the released gelatin in PBS containing the samples was estimated by **Bradford Assay.***

1. *firs calibration curve was prepared (0.05-1 g/l)*
2. *The samples were immersed in PBS at 37 for 24h.*
3. *Sample was collected and mixed with a Coomassi Blue reagent.*
4. *The absorbance was measured at 570 nm. The percentage loss of gelatin was examined by the formula:*

$$\%\text{Release} = \frac{100 \times C}{W_g \times 0.2}$$

Integral Stability Analysis

- *Key point is structure!*
- *IPN can be formed between gelatin and some polysaccharide.*
- *Physical IPN has a different structure from a normal IPN in which there are no direct crosslinks between the two networks.*
- *It is possible that the inter-network crosslinks are formed through intermolecular hydrogen bonding, ionic bonding, or physical entanglement.*
- *Ideal IPN is resulted by formation of both network efficiently (co-continues=no phase separation).*



Therefore, the ratio of 1:1 gives more ideal & densest IPN network → more integrated structure

Glucose Diffusion

- *It is important to understand the transport properties of these gels to predict if nutrients can freely enter the matrix, if desired cell products and cell waste products can freely be transported out of the matrix.*

Diffusion into the gel experiments:

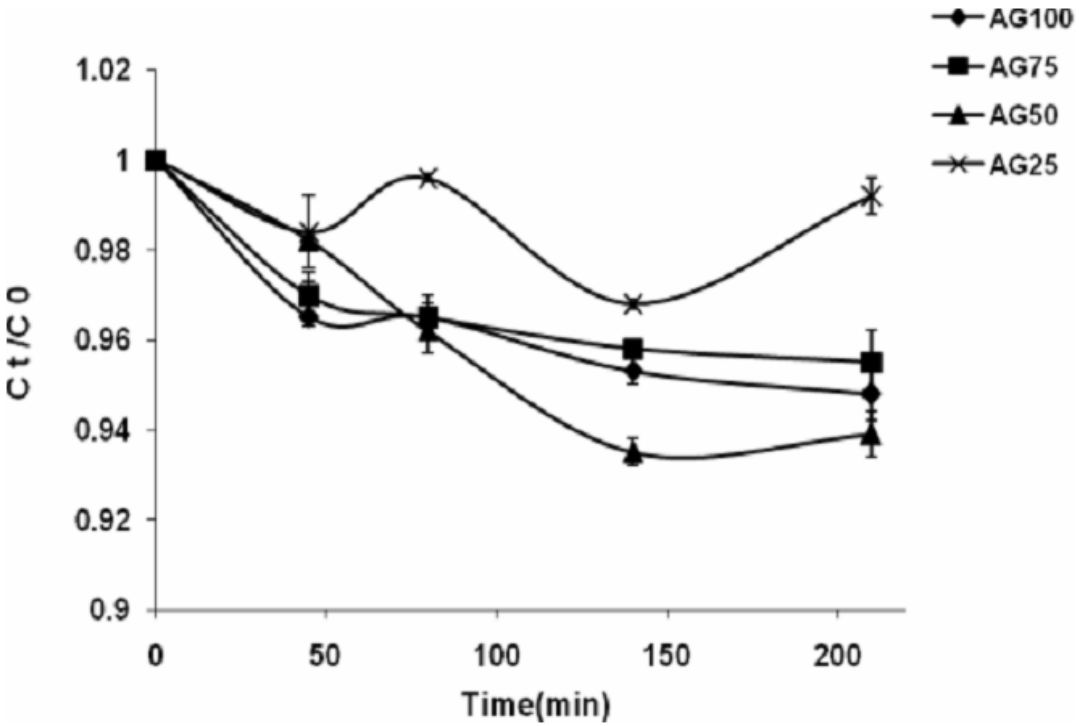
1. *Placing a single gel cylinder (3ml) in a 30-mL screw-cap glass vial filled with a 7mL solution of Glucose (2.5mg/ml).*

2. *Concentration changes to occur in the most accurate range as determined by biochemical autoanalyzer based on Glucose oxidase reaction*

3. *Monitoring was done in different time interval(45,80,240,210min)*

Glucose Diffusion

- *An unusual property of agarose gels is to behave like a sponge due to its porous nature.*
- *The agarose gels also allow diffusion of molecules which can be exploited for providing nutrients and gases to the cells entrapped within it.*
- *Blending causes some changes in the gel network structure, such as network density and pore size.*



Biocompatibility Analysis

- *Cytotoxicity of the blend hydrogels was evaluated by the MTT assay with PS tissue culture dishes as control.*

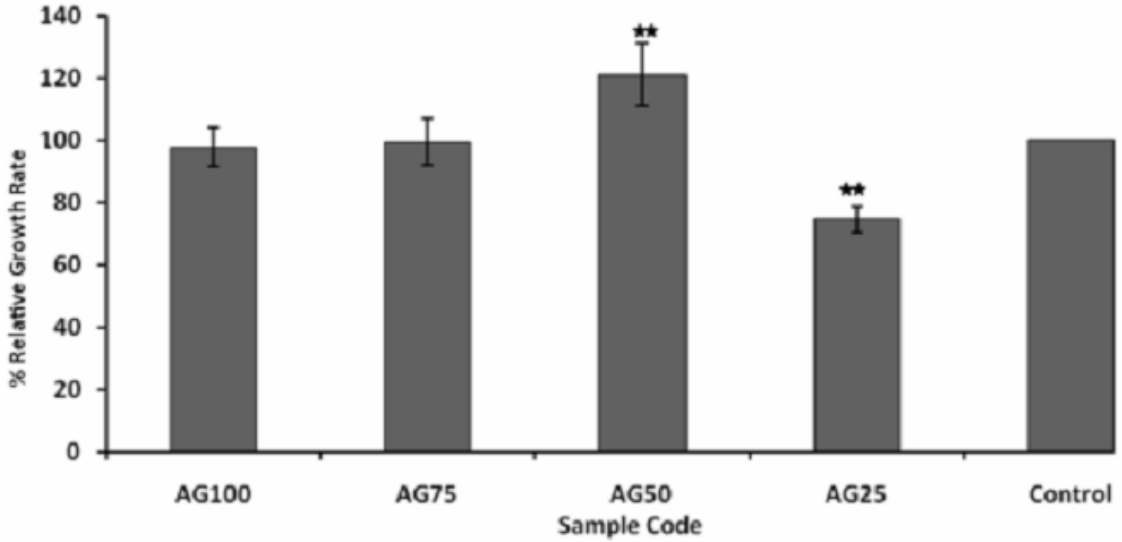
- *CHO cells were seeded on 96-multiwell at a density of 5000 cells/well.*
- *Following 24 h in culture at 37°C and 5% CO2, a layer of hydrogels added to each well*
- *Following another 48 h in cultures, cells were incubated in culture medium containing 1 mg/mL MTT solution.*
- *After incubation for 4 h, The absorbance of the solution was measured using ELISA reader at 570 nm.*

$$RGR = OD_e/OD_c \times 100$$

Relative growth rate

Biocompatibility Analysis

- *Agarose behaves more bioinert and gelatin more bioactive.*



The cell toxicity grade (CTG) of the AG100, AG75, and AG25 were grade 1 (75 < RGR < 99) indicating nontoxicity.

China Standard, GB/T 16886.5-1997, *Biological Evaluation of Medical Devices-Part 5: Tests for Cytotoxicity: In vitro methods*, **1997**.

Cell Attachment Analysis

- ***Qualitive study***

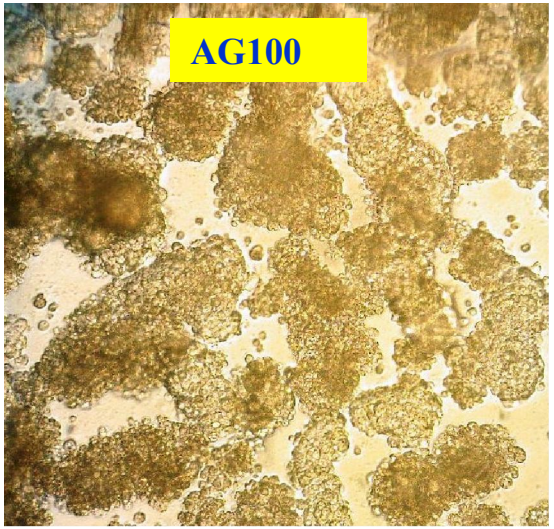
  Microscopic observation of cell morphology in contact with hydrogel surface

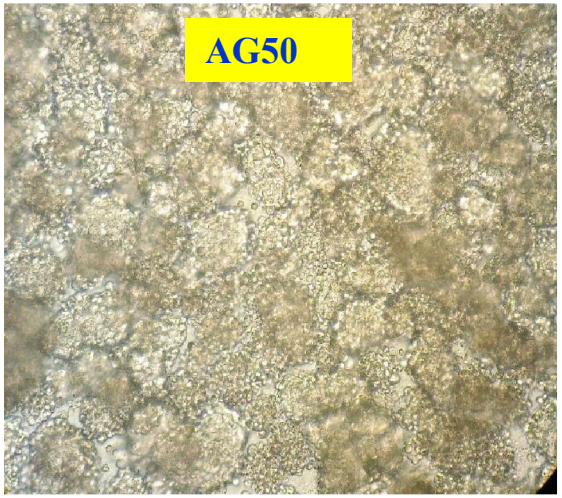
- ***Quantitive study***

  Unattached Cell counting Assay:

1. *The cell attachment studies were done on gelatin/ agarose hydrogel surface and culture plate dish as a control.*
2. *The 24-well plate culture was coated by 0.5 ml sterilized hydrogel.*
3. *1 mL cell suspension having cell density of 10_5 cells/ mL was loaded into each well. The plate was allowed to incubate*
4. *After 24 h the supernatant medium from each well containing unattached cells was carefully removed and the unattached cells were counted using hemocytometer.*

Cell Attachment Analysis





Sample Code	Average Unattached Cell Number	%Unattached Cell
AG100	92000	92
AG75	88000	88
AG50	46000	46
AG25	73000	73
Control	0	0

Conclusion: Part2

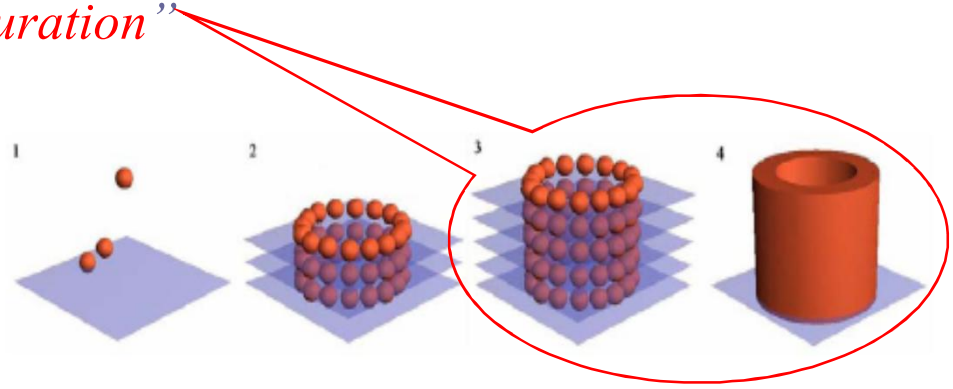
- *Determine of the optimum combination that can satisfy technical, biological, physical and mechanical requirements was aimed.*

According to the results:

- *Two samples: AG50 & AG75 could fulfill the requirements of a functional biopaper.*
- *Selection between them can based on objective tissue (mechanical properties and cell adhesiveness requirements)*
- *AG50 had more stable IPN like structure and showed more stability in physiological condition.*

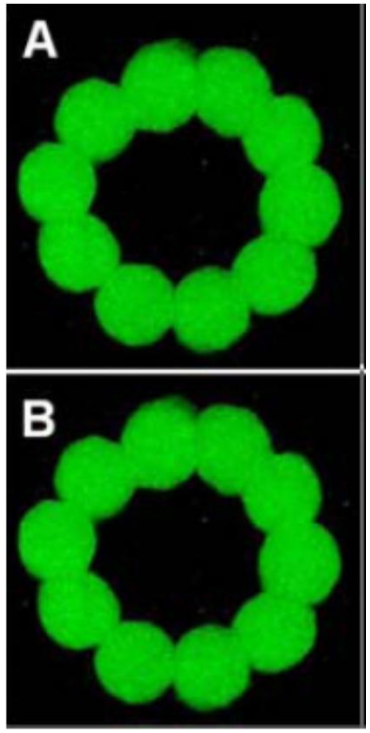
Tissue Fusion Ability & Kinetic

- *"Tissue Fusion" and "Tissue fluidity" is necessary for post-printing "Tissue Maturation"*



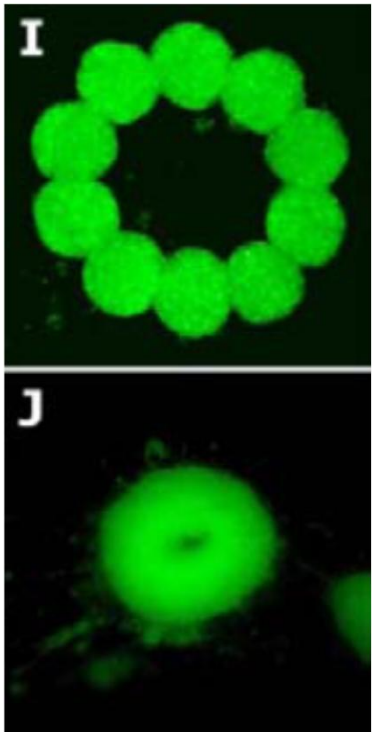
- *The ideal hydrogel for cell aggregate printing must provide favorable conditions for postprinting tissue fusion.*
- *The success of bioprinting hinges on the capability of the bio-paper and bioink to interact efficiently.*

Tissue Fusion Ability



Efficient Interaction?

Permissivity of hydrogel?



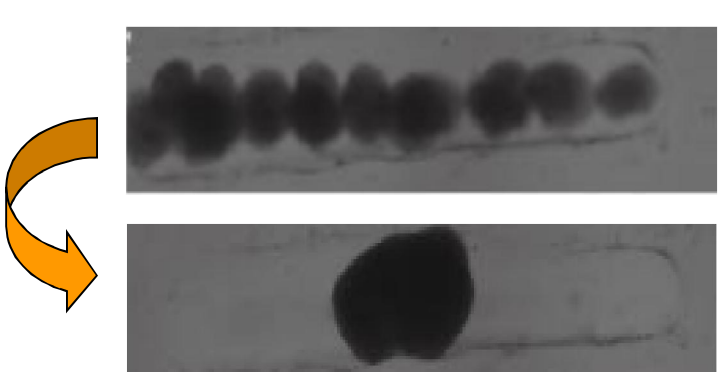
Agarose: non-permissive ☒

Collagen: so permissive ☒

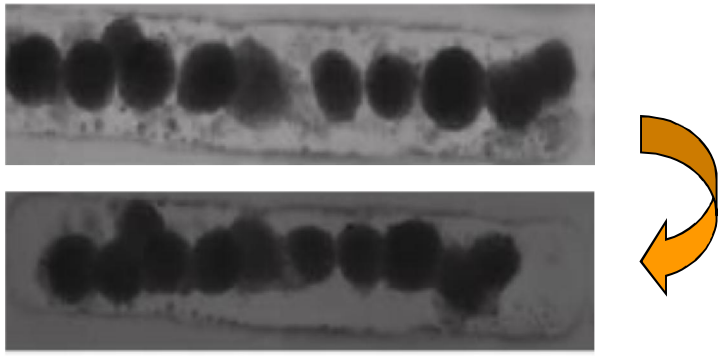
Tissue Fusion Ability

Efficient interaction?

Cohesivity of aggregate?



Less cohesive ☒

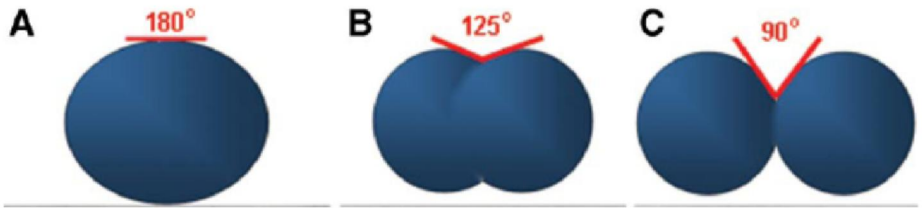


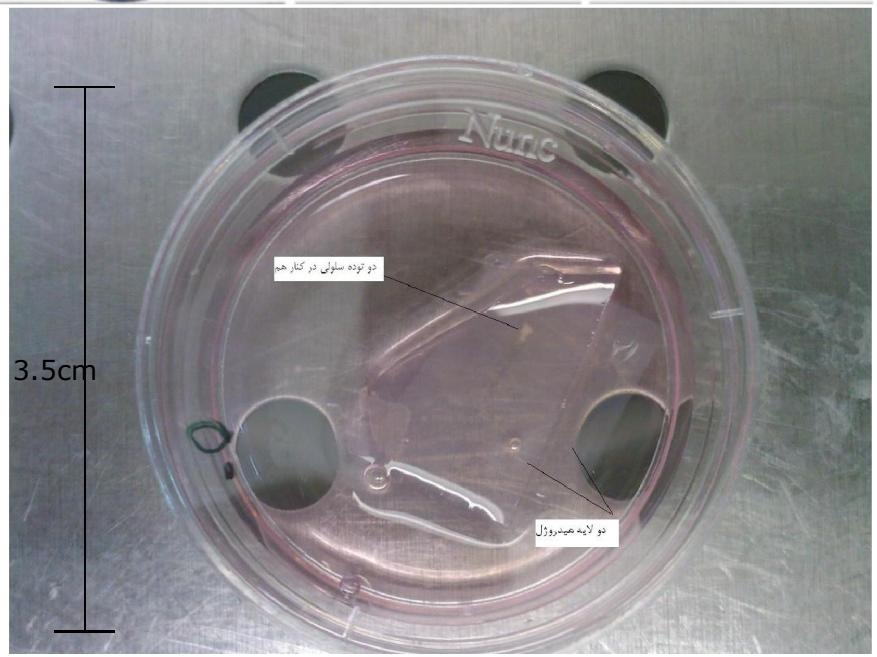
More cohesive ☒

Evaluation of Tissue Fusion Kinetic

Evaluation Tissue Fusion Kinetic:

Microscopic Observation & Angle Analysis



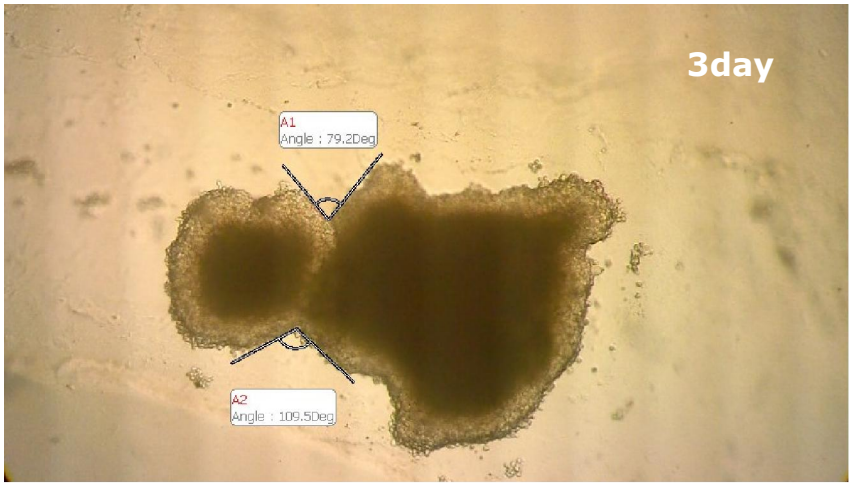


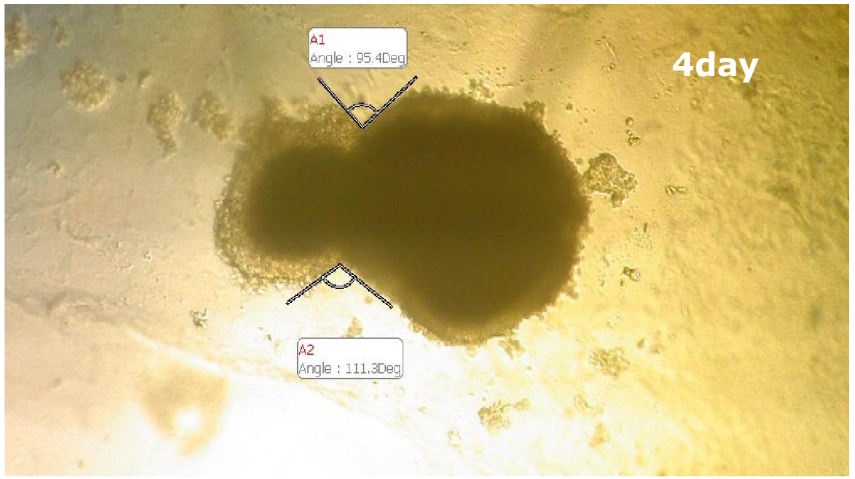
Bioink selection:

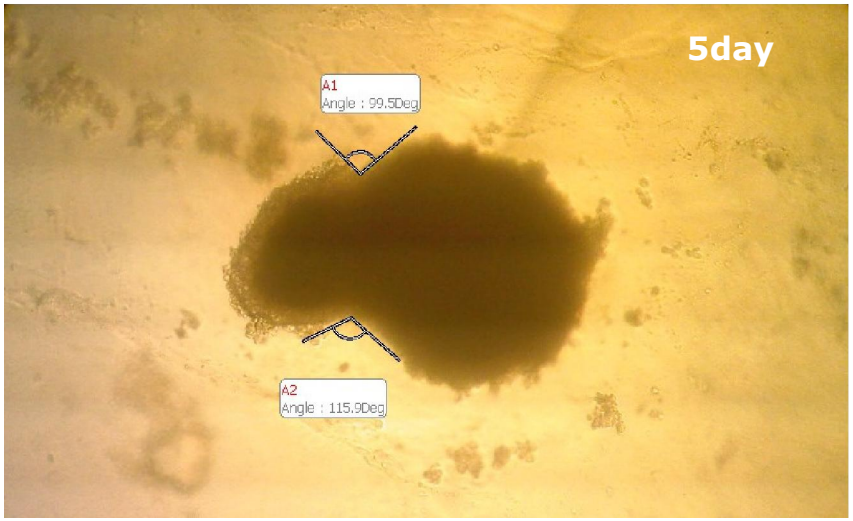
HD5

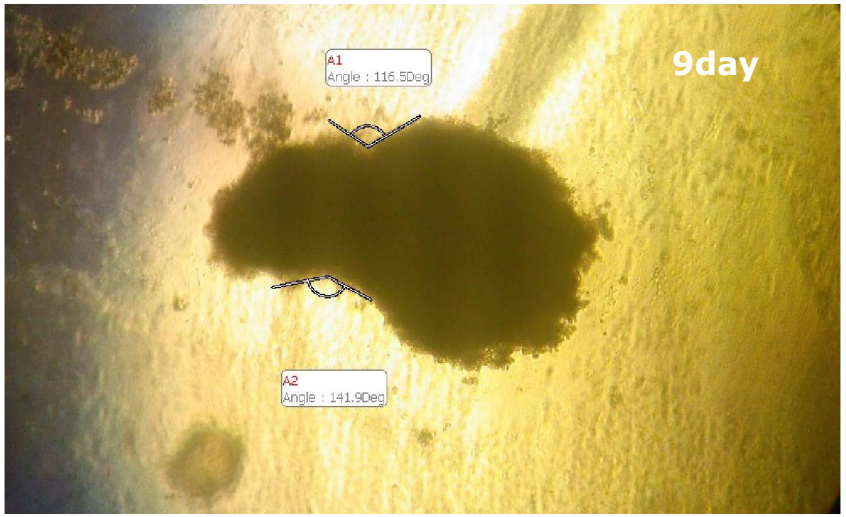
Biopaper selection:

AG50


3day
A1
Angle : 79.2Deg
A2
Angle : 109.5Deg


4day
A1
Angle : 95.4Deg
A2
Angle : 111.3Deg


5day
A1
Angle : 99.5Deg
A2
Angle : 115.9Deg

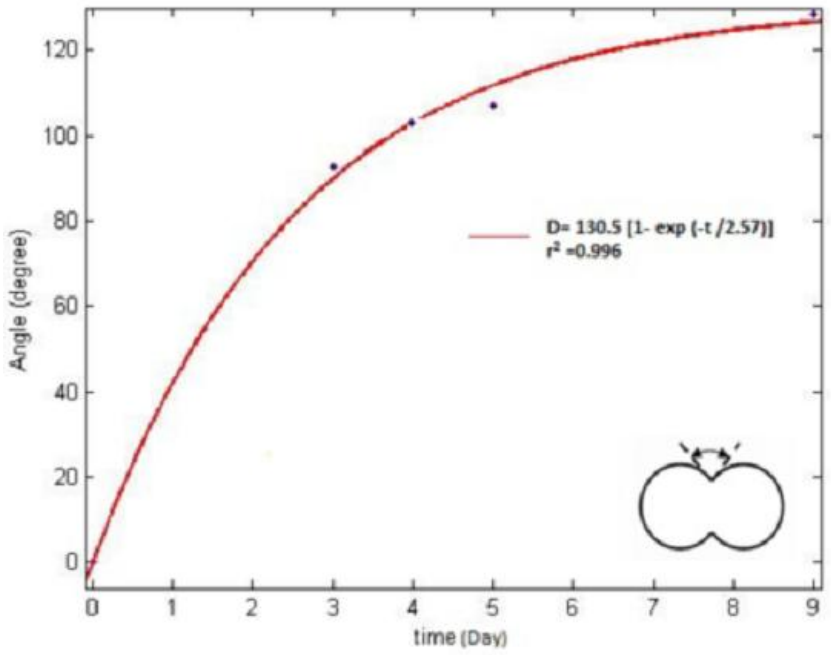

9day
A1
Angle : 116.5Deg
A2
Angle : 141.9Deg

Characterization of Fusion

$$D = C\,[1 - \exp(-t/\tau_{CC})]$$

A Positive Constant

Characteristic Timescale of the Aggregate Fusion



$\tau_{CC} \approx 2.5$day (this experiment)

$\tau_{CC} \approx 1$day for collagen(1mg/ml)

Comparison to collagen:

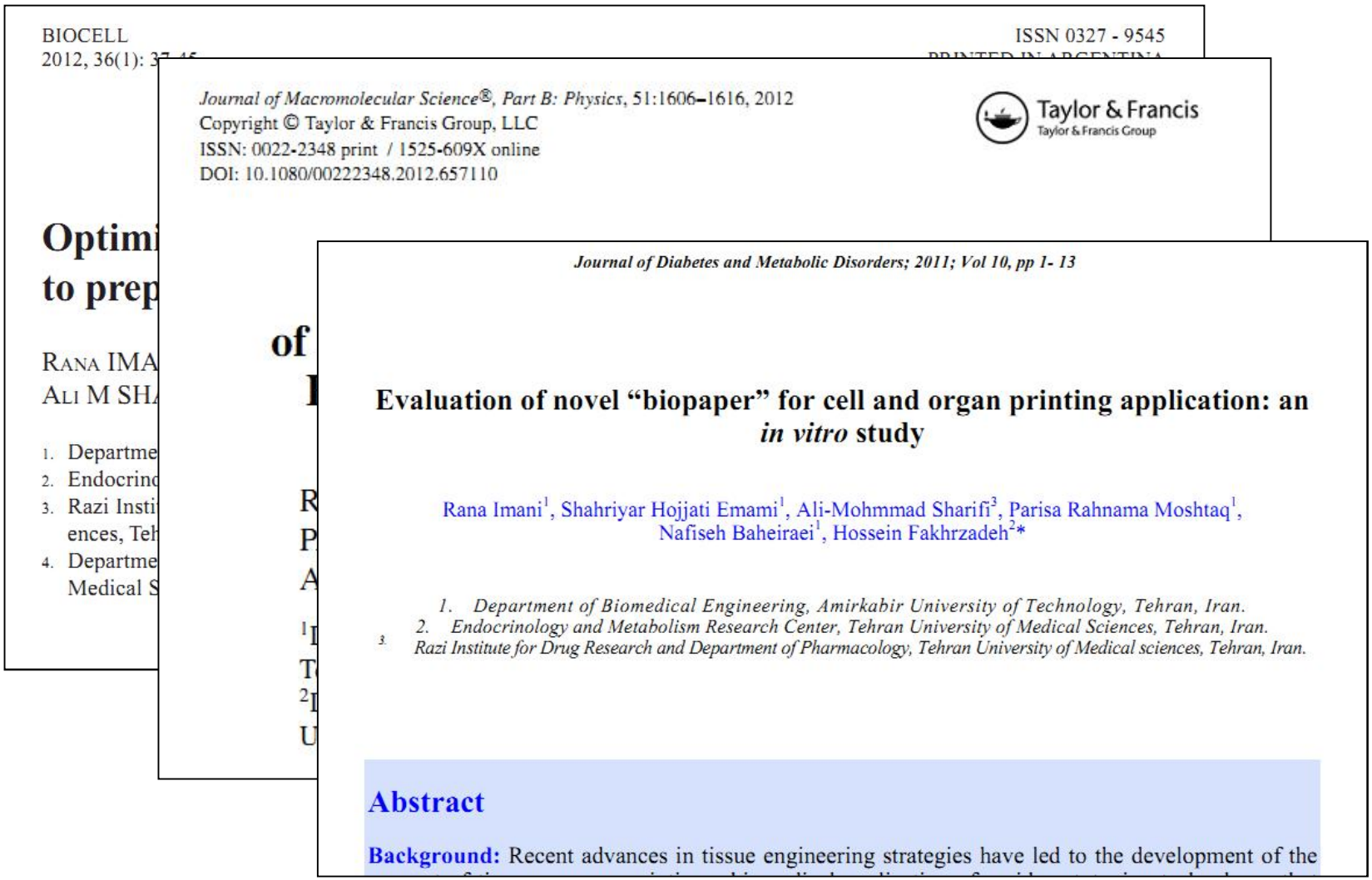
Aggregate has slower fusion rate and less compaction in AG50

Final Conclusion

- *This study tried to introduce a new vision of tissue engineering as a "Cell and Organs Bioprinting" that relies more on basic developmental biology.*
- *tissue fusion experiment, showed that combination of the same portion of agarose and gelatin (AG50) hydrogel could be expected requirements for a suitable and functional ink and paper.*
- *Aggregates with initial density of 5000 that underwent 3 days pre-culture showed suitable rate of tissue fusion.*

Since the **ultimate goal** tissue engineering is designing and constructing of body tissues similar to natural tissues, this goal will not be achieved unless by **understanding of** precise **mechanisms of natural evolution in the body tissues** and especially the formation of embryonic stages and close to the truth of **what normally happens in the human body.**

Results publications…



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Evaluation of novel "biopaper" for cell and organ printing application: an *in vitro* study

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3. *Razi Institute for Drug Research and Department of Pharmacology, Tehran University of Medical sciences, Tehran, Iran.*

Abstract

Background: Recent advances in tissue engineering strategies have led to the development of the

Acknowledgment

&
Special thanks

Thank you for your attention