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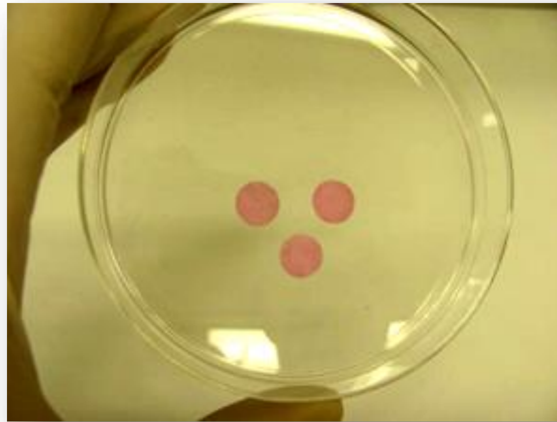
SEEDEZ™ PROTOCOLS

GEL EMBEDDING INTO THE SEEDEZ™

FOR PHYSIOLOGICALLY CLOSER EXTRACELLULAR ENVIRONMENTS IN 3D CELL CULTURES, INVASION, CHEMO-INVASION AND ANGIOGENSIS ASSAYS, AND



DRUG RELEASE STUDIES

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Gel Embedding into the SeedEZ™

December 2018

TABLE OF CONTENTS

INTRODUCTION ____ 1

Why to seed cells in a sol-state gel? ____ 1

The role of extracellular matrix ____ 2

Why to seed cells in a sol-state gel into the SeedEZ™? ____ 3

Why to embed a gel into the SeedEZ™ for cell motility assays, invasion and angiogenesis? ____ 4

Why to embed gel and other reagents into the SeedEZ™ for *in vitro* controlled drug release studies? ____ 5

GUIDELINES AND RECOMMENDATIONS FOR SEEDING CELLS IN A GEL ____ 6

GELS USED IN 3D CELL CULTURE APPLICATIONS AND DRUG DELIVERY ____ 6

Gels comprising extracellular matrix constituents ____ 6

Collagen I ____ 6

Matrigel ____ 7

Gels from natural sources for 3D cell cultures and controlled drug release ____ 8

Gels of animal origin ____ 8

Gelatin ____ 8

Gels of non-animal origin ____ 9

Agarose ____ 9

Alginate ____ 9

Methyl cellulose ____ 11

Synthetic gels ____ 12

Pluronic ____ 12

Carbomer ____ 12

INTRODUCTION

When compared to a 2D cell culture comprising one layer of *flat cells* on a non-physiologically stiff and flat Petri dish, three-dimensional (3D) cell culture provides tissue-like *round cell* morphology and tissue-soft cell environment with 3D cell-cell and 3D cell-extracellular matrix interactions, signaling, extracellular availability of soluble factors, oxygen etc.

SeedEZ™ is a substrate which makes transition to physiologically closer 3D tissue models easy. It makes the handling of 3D cell cultures as easy as handling a 2D culture so that you may continue with your protocols. It also makes 3D cell cultures consistent, even in high throughput and even if your 3D tissue models include extracellular matrices.

Extracellular matrix (ECM) is an important component of cell environment *in vivo*. ECM is a tissue-soft 3D support structure which holds cells together and to which cells adhere; a support structure which provides signaling molecules and diffusible guidance cues which influence cell fate. Cells respond to mechanical and biochemical changes in the ECM through the cross-talk between integrins and the actin cytoskeleton. *In vitro*, certain cell types are able to grow only if they are attached to surfaces comprising extracellular matrix (ECM) or its constituents.

SeedEZ™ allows incorporation of extracellular matrix into 3D culture models. It allows to seed cells in an ECM suspension, while providing lot-to-lot consistent cultures of defined dimensions in x, y, and z, and with reproducible cell and ECM gel distribution in 3D. With simple seeding methods and with its ability to wick viscous cell culture reagents, SeedEZ™ facilitates gel and cell handling to an extent that any user can generate *consistent* 3D gel cell cultures in minutes and in high-throughput. By allowing users to seed cells and extracellular matrix they choose, SeedEZ™ provides for the control of extracellular environment in which cells can be driven to better approximate function of their *in vivo* counterparts; thus, creating more robust, high-throughput and high-content assays.

Why to seed cells in a sol-state gel?

A. Most cells require cell-extracellular matrix interactions to maintain viability and function.

B. Not all cells secrete extracellular matrix (ECM) constituents they need, and they are surrounded by *in vivo*.

C. Many cells require specific ECM constituents to maintain their characteristic polarized organization, differentiated state and specific gene expression.

D. Many extracellular matrix proteins have binding sites for both cell adhesion and growth factors.

E. When cells are surrounded by an ECM and other cells in 3D, their morphology is closer to *in vivo,* and this influences virtually all cell functions.

F. An ECM gel *mimicking* biochemical and biomechanical cues present to cells *in vivo* is likely a better model of the extracellular environment than is the extracellular environment in which half the cell surface is exposed to medium and half-surface stuck to unphysiologically stiff plastic dish.

G. Soluble, diffusible guiding cues such as morphogen concentration gradients are difficult to establish, maintain and control in a 2D cell culture pool of medium.

H. ECM gel provides for mass transport of soluble factors and gas intra-3D-culture which is physiologically closer than is mass transport in a 2D culture in which half cell surface is bathed in medium and exposed to:

a. Abnormally high concentration of soluble factors.

b. Abnormally high oxygen tension.

c. Abnormal variations in extracellular conditions each time medium is changed (cells in a gel are "shielded" better).

I. The ECM gel stiffness and dimensionality have a profound effect on cell fate and function.

J. Extracellular matrix gel is the substrate for cell migration and invasion.

K. Cell movement and tissue remodeling are important for normal physiological processes and in pathology of diseases. For these processes to happen, the ECM must be present and frequently degraded for cells to migrate or to deposit a new matrix.

ECM PROVIDES FOR A MYRIAD OF IMPORTANT SIGNALS AND FUNCTIONS FOR RESIDENT CELLS, FUNCTIONS WHICH FLAT-CELL-ON-A-PLASTIC SURFACE MAY NOT BE ABLE TO SUBSTITUTE BUT ROUND-CELL IN AN ECM IN A 3D CELL CULTURE MIGHT BE ABLE TO. Recent studies have revealed the role of ECM in many pathological conditions, and ECM and its constituents have emerged as an important drug target.

"...It seems possible for cancer to be induced to become quiescent or revert to a normal state, if provided with the correct set of ECM signals, similarly to the normal embryogenesis."

Kim SH, Turnbull J, Guimond S.
Extracellular matrix and cell signalling: the dynamic cooperation of integrin, proteoglycan and growth factor receptor.
J Endocrinol. 2011, 209(2):139-51. Review.

Kenny PA, Bissell MJ.
Tumor reversion: correction of malignant behavior by microenvironmental cues.
Int J Cancer. 2003, 107(5):688-95.

The role of extracellular matrix

In vivo, extracellular matrix (ECM) is the scaffolding which holds cells together and includes collagen, elastin, proteoglycans, and glycoproteins such as fibronectin and laminin in varying composition depending on the tissue type. *In vitro* studies have shown that cell-ECM adhesion is not merely to immobilize cells. Cell-ECM signaling, and interactions bring about functional changes including cell survival, growth, proliferation, metabolism, extracellular availability of soluble and diffusible factors, migration, protein synthesis, and gene expression, all of which can be manipulated *in vitro* to mirror the *in vivo* conditions.

A large body of evidence suggests that cell adhesive molecules and biochemical cues in the extracellular environment influence cell function. Cell adhesion is mediated by specific receptors on the cell surface (integrins) which interact with the extracellular matrix constituents. Soluble and diffusible guidance cues in the ECM regulate cell fate. Biomechanical cues, mechanosensing and mechanotransduction are increasingly cited as important factors of the extracellular environment with profound effect on cytoskeletal organization and cell function. Cells sense stiffness, topography and dimensionality of the extracellular environment through a process called mechanosensing which influences cell signaling, function and fate. Cells respond to biomechanical properties of the environment through a process called mechanotransduction by altering their organization, traction, protein expression, motility, etc. Clearly, the role of ECM on cell fate is profound.

In a physiologically closer tissue model, ECM needs to be present and cell-ECM cross-talk needs to be present. SeedEZ™ allows effortless incorporation of the ECM into high-throughput cell-based assays.

References:

Fischer RS, Myers KA, Gardel ML, Waterman CM.
Stiffness-controlled three-dimensional extracellular matrices for high-resolution imaging of cell behavior.
Nat Protoc. 2012, 7(11):2056-66.
http://www.ncbi.nlm.nih.gov/pubmed/23099487

Wells RG.
The role of matrix stiffness in regulating cell behavior.
Hepatology. 2008, 47(4):1394-400.
http://www.ncbi.nlm.nih.gov/pubmed/18307210

Yeung T, Georges PC, Flanagan LA, Marg B, Ortiz M, Funaki M, Zahir N, Ming W, Weaver V, Janmey PA.
Effects of substrate stiffness on cell morphology, cytoskeletal structure, and adhesion.
Cell Motil Cytoskeleton. 2005, 60(1):24-34.
http://www.ncbi.nlm.nih.gov/pubmed/15573414

Yamada, K. M.
Extracellular Matrix.
Current Protocols in Cell Biology. 2009, 45:10.0.1–10.0.3.
http://onlinelibrary.wiley.com/doi/10.1002/0471143030.cb1000s45/pdf

Why to seed cells in a sol-state gel into the SeedEZ™?

While seeding cells in a sol-state gel is a standard technique, it requires skill. Without automated equipment, 3D gel culture shape is often inconsistent culture-to-culture and culture dimensions in x, y, and z are difficult to control at seeding. For those gels which gelation is initiated with rise in temperature, cultures tend to spread out in routine transfer to incubator for gelling. In essence, they flatten out even before they are gelled. In chemically cross-linked hydrogels, cells are seeded in a precursor. Unfortunately, cells settle during the process of cross-linker addition to cultures in different wells. All this yield questionable 3D cell cultures, with significant variations in x, y and z dimensions, and cell and gel distribution in x, y, and z at seeding or following gelling.

Inconsistent culture dimensions and cell distribution across these dimensions at seeding yield culture-to-culture varying supply of nutrients, removal of catabolic waste products, and intra-culture concentrations of trophic factors, autocrine and paracrine signaling molecules cells secrete to regulate their environment, growth and many other functions. Some signaling molecules degrade quickly, limiting the scope of their effectiveness to the immediate cell surroundings. Others affect only nearby cells because they are taken up quickly, or because their transport is hindered by the extracellular matrix. In sum, inconsistent cultures at plating yield inconsistent tissue analogs for life sciences research and drug testing. Variations in cell function and secretion of drug metabolizing enzymes, as well as variations in intra-culture availability of test compounds influence pharmacokinetic studies, resulting in less conclusive cell outcomes and problems with interpretation of results.

SEEDEZ™ SOLVES THESE PROBLEMS AND TRANSFORMS 3D GEL CULTURES INTO **CONSISTENT 3D CELL CULTURES** WHICH ARE EASY TO SEED AND EASY TO HANDLE IN HIGH-THROUGHPUT.

IF EMBEDDED IN THE SEEDEZ™, 3D GEL CULTURES CANNOT BE ACCIDENTALLY ASPIRATED OR DETACHED IN ROUTINE MEDIA EXCHANGES. THIS ALLOWS REPRODUCIBLE STUDIES TO CONTINUE DAYS AND WEEKS AFTER CELL SEEDING.

With simple seeding methods and with its ability to wick viscous reagents, SeedEZ™ provides remarkable experimental flexibility to embed cells, gels, and compounds of choice in order to, for example, model stiff

extracellular matrices in aging tissues with important ramifications to cell fate and potentially higher relevance of the *in vitro* compound screening results.

Additional advantage of SeedEZ™ is that it provides 3D cell support even when a gel degrades. When gel dissolves or thins down due to enzymatic or hydrolytic degradation, cell digestion, or other factors, cells remain supported in 3D by the SeedEZ™. If your studies cannot be completed, are too short to answer specific questions, or simply difficult to repeat due to inconsistent and time-dependent gel decay, SeedEZ™ can help. Gels which degrade too quickly may not allow cells to mature and form functional networks for compound screening. On the other hand, gels which are difficult to remodel, may prevent cells from invading or depositing their endogenous matrix constituents. In both cases, SeedEZ™ is the solution. When gel degrades too fast, SeedEZ™ continues to support cells. If gel concentration is too high, SeedEZ™ may enable use of a lower concentration gel which is still held in 3D by the SeedEZ™.

Why to embed a gel into the SeedEZ™ for cell motility assays, invasion and angiogenesis?

A. In a truly 3D cell migration, representative of *in vivo* conditions, invasive cells are embedded in the extracellular matrix from the assay start to its end.

Standard trans-membrane cell assays study cell invasion in an environment that is quite different from that *in vivo*. Cells are plated in a monolayer at the liquid-ECM interface, and the trans-membrane is permissive only to unidirectional (vertical) cell migration through the membrane pores.

The key difference between a thin layer of ECM on a membrane and the ECM in the SeedEZ™ is that the migration/invasion in the SeedEZ™ is truly 3D; cells are embedded in 3D and migrate in 3D which is closer to in vivo conditions.

B. Physiologically closer extracellular environment with respect to ECM composition, gel stiffness, and the presence of diffusible or substrate-bound cues, for physiologically closer assay results.

Emerging evidence elucidates role of physical resistance present to cells when migrating and invading in 3D, a resistance which is often negligible in 2D but present *in vivo*. In 3D, ECM resistance forces cells to develop proteolytic and amoeboid (non-proteolytic) strategies to either degrade the matrix to migrate (proteolytic migration) or to "deform and squeeze" (amoeboid migration) through the 3D ECM.

SeedEZ™ allows embedding of most ECMs at high protein concentration to study cell invasion in 3D in a physiologically closer setting with respect to biochemical and biomechanical cues present to cells taxing or invading in 3D. Putative modulators of cell motility, invasion, angiogenic inhibitors etc., can all be tested using Spot-an-Agent and Spot-a-Culture™ approach. This may be done in "side-by-side" testing in one SeedEZ™ substrate, or in a SeedEZ™ sandwich or stack.

C. Cell-based assays with preserved cell morphology, heterogeneity of cell types, cell-cell and cell-ECM interactions and signaling, and with extracellular cues having sufficient specificities to model pathologies in question may be able to provide physiologically closer cell-based assay results.

Normal and malignant cells can be distinguished morphologically in 3D environments. Solid tumors have pronounced cellular heterogeneity on the histological, genetic, and gene expression level. Cells differ in size, morphology, motility, angiogenic, proliferative, invasive potential, and drug resistance. Still, the majority of invasion assays still employ a single cell type, and even for that cell type its size has to "match" the pore size on the trans-membrane to even conduct an assay.

SeedEZ™ allows to seed heterogeneous cell populations and sub-populations for physiologically closer tissue modeling and 3D cell-based assay development.

D. Gel deteriorates too fast in angiogenesis/ transmigration assays.

SeedEZ™ solves this problem because it continues to support cells even when a gel degrades and allows embedding of high protein gels. Substrates may also be stacked for consistent and reproducible longer-term studies.

References:

Ehrbar M, Sala A, Lienemann P, Ranga A, Mosiewicz K, Bittermann A, Rizzi SC, Weber FE, Lutolf MP.
Elucidating the role of matrix stiffness in 3D cell migration and remodeling.
Biophys J. 2011, 100(2):284-93.
http://www.ncbi.nlm.nih.gov/pubmed/21244824

Brekhman V, Neufeld G.
A novel asymmetric 3D in-vitro assay for the study of tumor cell invasion.
BMC Cancer. 2009, 9:415.
http://www.ncbi.nlm.nih.gov/pubmed/19948022

Albini A, Benelli R, Noonan DM, Brigati C.
The "chemoinvasion assay": a tool to study tumor and endothelial cell invasion of basement membranes.
Int J Dev Biol. 2004, 48(5-6):563-71.
http://www.ncbi.nlm.nih.gov/pubmed/15349831

Bissell MJ, Rizki A, Mian IS.
Tissue architecture: the ultimate regulator of breast epithelial function.
Curr Opin Cell Biol. 2003, 15(6):753-62.
http://www.ncbi.nlm.nih.gov/pubmed/14644202

Marusyk A, Polyak K.
Tumor heterogeneity: causes and consequences.
Biochim Biophys Acta. 2010, 1805(1):105-17.
http://www.ncbi.nlm.nih.gov/pubmed/19931353

Why to embed gel and other reagents into the SeedEZ™ for *in vitro* controlled drug release studies?

Many inert polymers (gels) have been used as drug delivery systems both *in vitro* and *in vivo*. By incorporating drugs into the SeedEZ™ or in biodegradable polymer matrices into the SeedEZ™, the dosage forms which release a drug over a period of time may be prepared in a desired shape and size without complex protocols and special equipment. More importantly, all SeedEZ™-based dosage forms can be tested *in situ*, with the cells in a 3D cell culture. Rigid, yet absorbent SeedEZ™ wicks diverse sol-state gels, pharmaceuticals and test compounds. As a result, SeedEZ™ offers a novel 3D framework for:

A. Development of sustained release drug delivery systems that are simple and convenient to use *in vitro*.

B. *In situ* testing of the above drug delivery systems with 3D cultures.

C. Localized or distributed drug delivery into 3D cultures using Spot-a-Culture™ and Spot-a-Drug™ method in a single substrate or in a SeedEZ™-stack.

D. Development of test platforms for quasi-steady drug release.

   In diffusion driven drug delivery systems, drug release rate often declines in time. A BIODEGRADABLE POLYMER MATRIX EMBEDDED IN THE SEEDEZ™ MAY ENABLE QUASI-STEADY DRUG RELEASE FROM A DEFINED VOLUME, DEFINED BY THE SEEDEZ™, WHEN THE EMBEDDED MATRIX DEGRADATION RATE IS ADJUSTED TO COMPENSATE FOR THIS DECLINE WITH AN INCREASED DRUG PERMEABILITY FROM THE SYSTEM.

GUIDELINES AND RECOMMENDATIONS FOR SEEDING CELLS IN A GEL

While the SeedEZ™ allows embedding of sol-state and even semi-sol-state gels from various origins and sources, for the most consistent results embed gel in as much sol state as possible. This may depend on the nature of the gel; for example, if the gel gels via physical or chemical cross-linking methods:

1. For temperature-dependent hydrogels, and thermo-reversible hydrogels dispense at a suitable temperature when the gel is in sol-state provided that that temperature is not detrimental to cells seeded in the gel.

2. For chemically cross-linked hydrogels, dispense, wick or dip a gel precursor first, if possible, and then add cross-linking agent when precursor is embedded in the SeedEZ™.

For example, for Alginate gels, you may embed cells in sodium alginate into the SeedEZ™ followed by addition of Calcium ions in a buffer to crosslink the gel precursor at close to physiological conditions (or dip SeedEZ™ into Calcium containing buffer), followed by 3D culture incubation in medium.

Pipette sol-state gel solutions carefully to avoid bubble formation. Use a positive displacement micropipette to dispense viscous solutions, or if you find that losses in pipetting are significant using an ordinary micropipette. In some cases, concentrated sol-state gels may be too viscous to dispense even with a positive displacement pipette. In this event, use dip-in method to embed gel into the SeedEZ™. Dip-in method works for many gels even when the gel viscosity is approximately half the honey-like viscosity.

Most commonly, cells are seeded in a sol-state gel into the SeedEZ™. This provides truly 3D cell distribution at seeding. If the objective is to seed cells on top of a gel embedded in the SeedEZ™, you may still do so; however, you may need to centrifuge the plate to make cells ingress into the gel. To achieve desired culture conditions at seeding, you may also overlap or stack the SeedEZ™ substrates, or you may add a layer of ECM between two SeedEZ™ substrates which already comprise the same or a different gel.

GELS USED IN 3D CELL CULTURE APPLICATIONS AND DRUG DELIVERY

MOST GEL FORMING POLYMERS ARE SUSCEPTIBLE TO DEGRADATION BY REACTIVE OXYGEN SPECIES. THE USE OF SEEDEZ™ IS RECOMMENDED WITH MOST GELS USED IN 3D CELL CULTURE APPLICATIONS, ESPECIALLY IF CELLS DO NOT SECRETE THEIR ENDOGENEOUS EXTRACELLULAR MATRIX COMPONENTS THEY NEED TO MAINTAIN VIABILITY AND FUNCTION.

GELS COMPRISING EXTRACELLULAR MATRIX CONSTITUENTS

COLLAGEN I

Collagen Type I is a fibrillar protein which provides structural support to cells and facilitates cell attachment, growth, differentiation and migration.

When used as a gelled matrix to support cells in 3D, Collagen Type I derived from different sources may

Fig. 1 Collagen Type I, gel embedded in the SeedEZ.
Without pre-wetting treatments, the SeedEZ instantly wicks Bovine Collagen Type I, BD Biosciences Product No. 354231, at a protein concentration of 2.9 mg/ml. According to the supplier, under suitable conditions, this product gelled in solutions containing as little as 0.5 mg/ml of protein.

not gel equally fast. For example, Bovine Collagen may gel slower than a Rat Tail Collagen. In general, a faster gelling gel allows cells to remain better distributed in 3D, but the working time may be short, while in slow-to-gel gels cells tend to settle. The latter is of concern if you are seeding cells in a sol-state gel (without the SeedEZ™) with the objective of obtaining 3D cell distribution.

SeedEZ™ accepts Collagen I as a sol-state gel (Fig. 1). With the SeedEZ™, you may find that cell distribution is more uniform in 3D even with Bovine Collagen I. This is because SeedEZ™ opposes cell settling, instantly wicks sol-Collagen, and may be kept at 37 ^{o}C prior to cell seeding in Collagen suspension to accelerate its gelation. When preparing Collagen I for use with 3D cell cultures, follow manufacturer protocols or other protocols you normally use.

Among cells cultured in or on Collagen I are primary colon carcinoma cells, mouse liver progenitor cells, rat pancreatic islet cells, endothelial cells, hepatocytes, muscle cells, breast carcinoma cells, osteoclasts, and transfected cell lines including NIH3T3, PC12, and HEK-293. The type of Collagen used, its concentration, cell types, cell seeding method, and a culturing period depend on application and research objectives. In general, cells may be seeded distributed in Collagen in the SeedEZ™, seeded on top of the Collagen in the SeedEZ™, or seeded between two or more layers of Collagen embedded in two or more SeedEZ™ substrates.

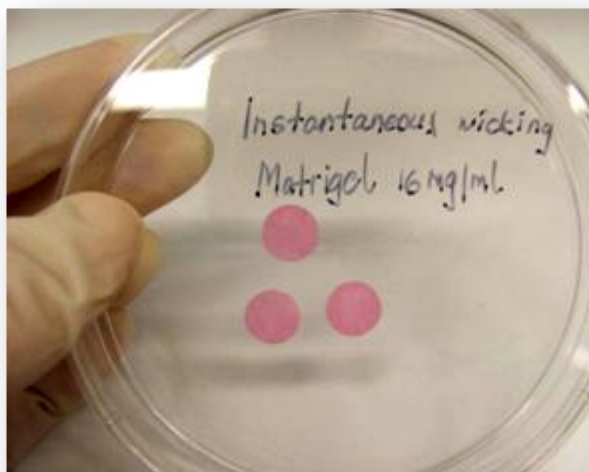

Fig. 2 Matrigel gel (16 mg/ml) embedded in the SeedEZ. Without pre-wetting treatments, the SeedEZ instantly wicks Growth Factor Reduced Matrigel, diluted to 16 mg/ml in HBSS (from the high protein concentration stock); BD Biosciences Product No. 354263.

MATRIGEL

Matrigel is a gelatinous protein mixture secreted by Engelbreth-Holm-Swarm mouse sarcoma cells (EHS matrix). This ECM resembles extracellular environment in many tissues and is used by cell biologists as a gelled extracellular matrix for 3D cultures. Main components of Matrigel are structural proteins (Laminin, Collagen IV and Entactin). Matrigel promotes and maintains differentiated phenotypes in diverse cultures.

SeedEZ™ accepts Matrigel (Fig. 2) delivered as a sol-state gel up to 16 mg/ml protein concentration (the highest protein concentration tested). According to manufacturer, Matrigel is a physiologically relevant surface for many applications and cell types, including human embryonic and induced pluripotent stem cells, myogenic cells, mammary epithelial cells, hepatocytes, rat brain microvessels, mammary acinar formation, and endothelial tube formation.

GELS FROM NATURAL SOURCES FOR 3D CELL CULTURES AND CONTROLLED DRUG RELEASE

Hydrocolloidal materials derived from natural sources are fully or partially soluble in water and used as gelling agents in 3D cell culture applications. Commonly, they are either protein-based or polysaccharide-based biomaterials. An example of gel derived from animal proteins is gelatin. Polysaccharide-based polymers represent a large class of biomaterials used in 3D cell culture applications including agarose, alginate, carageenan, dextran, chitosan, cellulose derivatives etc.

THESE BIOMATERIALS ARE ALSO USED IN PREPARATION OF DRUG DELIVERY SYSTEMS (ALGINATES, GELATINS ETC.), OR USED AS SUBSTRATES FOR CONTROLLED DRUG RELEASE (CHITOSAN, CELLULOSE DERIVATIVES, AGAROSE ETC.).

SeedEZ™ allows embedding of most sol-state gels while providing a convenient 3D framework for cell cultures studies, cell-based assay development, drug release studies and other applications.

GELS OF ANIMAL ORIGIN

GELATIN

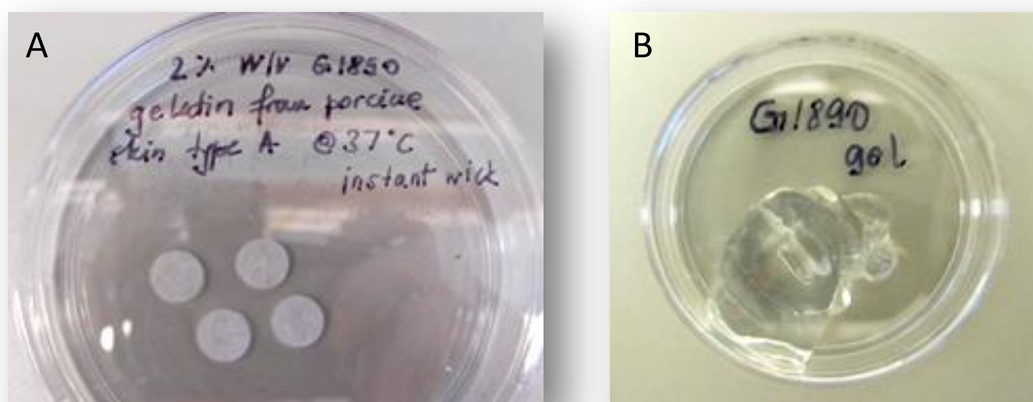

Fig. 3 Type A gelatin embedded in the SeedEZ.

A. Without pre-wetting treatments, the SeedEZ instantly wicks porcine skin gelatin; a 2% w/v solution delivered to the SeedEZ substrates at 37ºC. Type A indicates that the gelatin was derived from acid cured tissue, Sigma-Aldrich Product No. G1890.

B. Room temperature gelled Gelatin; the same as delivered to the SeedEZ substrates in **A**.

Gelatin is a mixture of water-soluble peptides and proteins produced by partial hydrolysis of Collagen. It provides an attachment and growth promoting substrate for the culture of many cell types. Gelatin is a thermo-reversible hydrogel suitable for 3D cell cultures either alone or in combination with other molecules.

When an aqueous solution of gelatin at a concentration > 0.5% is cooled to 35-40°C it first increases in viscosity and then gels. The gel stiffness depends on gelatin composition, method of manufacture, thermal history, concentration in solution, pH and temperature. The optimal gel stiffness depends on cell type, application and the research objectives. In addition to thermally-cooled or physically cross-linked gelatin gel, gelatin may be also cross-linked chemically:

Yung CW, Wu LQ, Tullman JA, Payne GF, Bentley WE, Barbari TA.

Transglutaminase crosslinked gelatin as a tissue engineering scaffold.

J Biomed Mater Res A. 2007, 83(4):1039-46.

Gelatin is biodegradable, thermally degradable, and susceptible to hydrolysis. SeedEZ™ accepts gelatin as a sol-state gel (Figs. 3-4) and helps with its application in long-term cell culture studies.

GELS OF NON-ANIMAL ORIGIN

AGAROSE

Agarose is a linear polysaccharide derived from agar, a hydrophilic colloid which forms thermo-reversible gels. In its gelled state, agarose is used as matrix for 3D cell aggregates (spheroids) and 3D cell cultures of dissociated cells. Agarose melts at higher than physiological temperatures and gels at temperatures close to 37°C. This makes it suitable for cell-based assay development, molecular biology applications and drug release studies. The use of agarose as a matrix through which chemoattractants diffuse provides a system to study cell motility using the "agarose drop" assay, the "agarose plug" assay, and the "under agarose" assay.

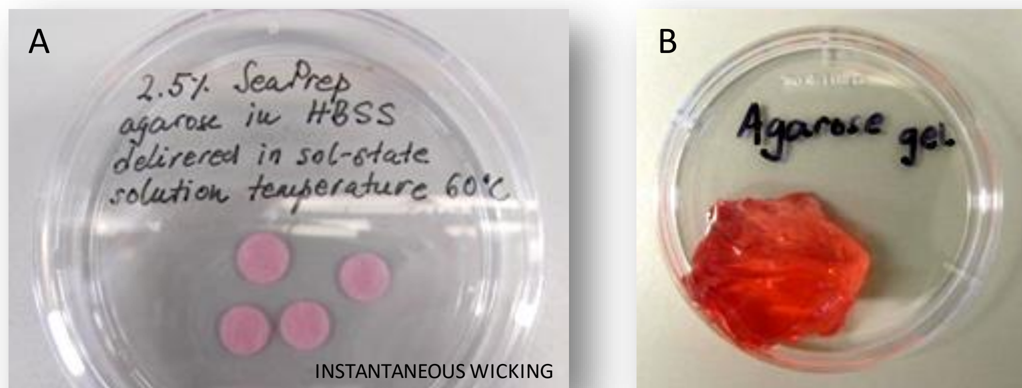

Fig. 5 Agarose gel embedded in the SeedEZ.

A. Without pre-wetting treatments, the SeedEZ instantly wicks SeaPrep Agarose, Lonza Rockland Product. No. 50302; a 2.5% w/v solution delivered to the substrates at 60ºC.

B. Room temperature gelled agarose; the same as delivered to the SeedEZ substrates in **A**.

Stiffness and porosity of agarose gels depend on agarose concentration in solution, while gelling and melting temperatures may be adjusted by controlled introduction of hydroxyethyl groups. Agarose gels are frequently used as matrices for 3D cell cultures at concentrations higher than about 2% w/v.

SeedEZ™ accepts sol-state agarose gel (Fig. 5). Most commonly, agarose is used to support 3D cell cultures of cartilage phenotype. With covalently coupled Laminin, agarose gels were found to significantly enhance neurite extension from 3D cultured embryonic day 9 chick dorsal root ganglia, and PC 12 cells.

ALGINATE

Alginates are hydrocolloids, water-soluble biopolymers produced by brown seaweeds. They are often supplied as sodium salts which are soluble in water. In the presence of divalent cations, sodium alginates form cross-linked gels. Dissociated cells and 3D cell spheroids are seeded in sodium alginate, followed by dipping or short-term incubation in a Calcium buffer at close to physiological conditions. This procedure yields a cross-linked gel in which cells are embedded. Cells may be also encapsulated in alginate beads or microcarriers using the same approach. Unlike ECM proteins, alginate is an inert material lacking Arg-Gly-Asp peptide sequence which enables cell anchorage through integrins. Alginate is often used to culture cell types that

aggregate and form 3D cell spheroids. However, covalently bound peptides (integrin binding ligands) may be necessary for the culture of anchorage dependent cells which do not aggregate to model cell-matrix interactions.

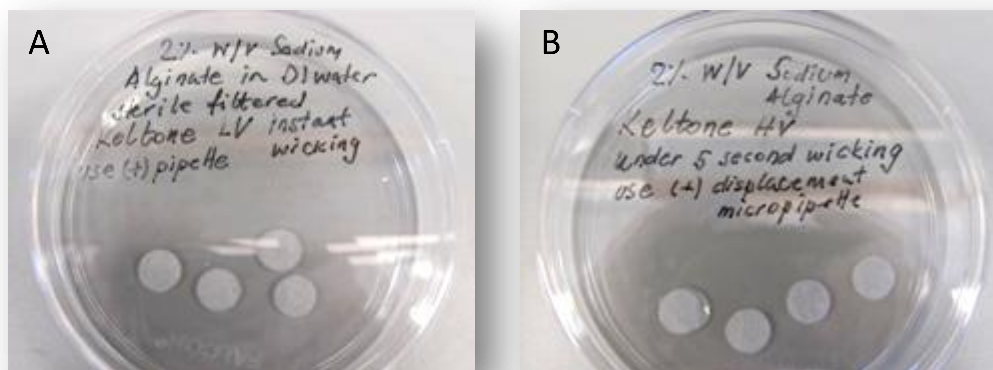

Fig. 6 Sodium alginate embedded in the SeedEZ.

A. Without pre-wetting treatments, the SeedEZ instantly wicks sodium alginate Keltone LVCR, FMC Biopolymer; a 2% w/v solution delivered using a positive displacement pipettor.

B. Without pre-wetting treatments, the SeedEZ SC-C048 wicks sodium alginate Keltone HVCR, FMC Biopolymer; a 2% w/v solution in less than 10 seconds.

SeedEZ™ wicks viscous sodium Alginate solutions 2% w/v (Fig. 6). The two sodium alginates tested were Keltone LVCR and Keltone HVCR. Both products are used as drug-polymer matrices for extended release (polymer matrices for tablets used in orally administered controlled drug delivery) and polymer matrices for 3D cell cultures or spheroids (for neural stem cell growth, embryonic stem cell growth, or chondrocytes in alginate gels or beads). According to supplier, viscosity of a 2% Keltone LVCR solution is 100-300 cPs. The viscosity of a 1.25% Keltone HVCR solution is 600-900 cPs; a 2% solution was used for wicking into the SeedEZ™. For reference, at room temperature, viscosity of water is 1 cP and that of honey about 10,000 cPs. Solutions were delivered to the SeedEZ™ substrates using a positive displacement pipette. For higher viscosity alginate (Keltone HVCR), dip-in method was more appropriate.

At a concentration higher than 1 % w/v, sodium alginates may be too viscous and difficult to dispense consistently culture-to-culture. This is inconvenient for 3D cell cultures where cell suspensions at seeding and following seeding may be less homogeneous with the corresponding non-uniformity in 3D cell distribution. The SeedEZ™ solves this problem and wicks sol-state 2% w/v viscous sodium alginate solutions by spot- and dip-in method (Fig. 6). As the SeedEZ™ holds cells in 3D by itself, it also enables the use of lower concentration alginates for less constrained cell growth. Alginate gels degrade by methods which reverse gelation. As the gel disintegrates due to gradual exchange of Calcium ions with Sodium, the use of SeedEZ™ is recommended, particularly if low concentration gels were used.

METHYL CELLULOSE

Cellulose is the most abundant naturally occurring polymer of glucose. Cellulose-based hydrogels are formed by cross-linking aqueous solutions of cellulose ethers such as methylcellulose, hydroxypropyl methylcellulose, ethyl cellulose, hydroxyethyl cellulose and sodium carboxymethylcellulose. Thermo-reversible gels are prepared from aqueous solutions of methylcellulose and/or hydroxypropyl methylcellulose. While up to 10% methylcellulose solutions can be prepared using low-viscosity products (< 10-50 cPs), the high-viscosity methylcellulose solutions are normally limited to 2-3%.

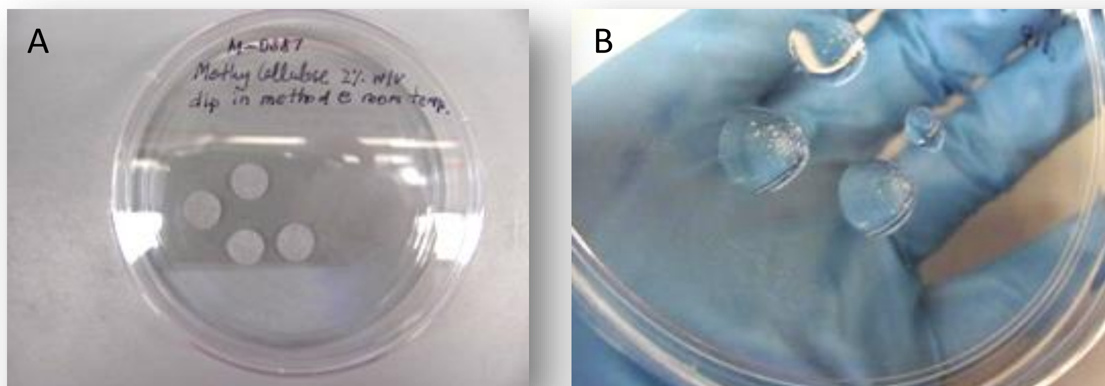

Fig. 7 Methylcellulose semi-solid media embedded in the SeedEZ.

A. Without pre-wetting treatments, the SeedEZ accepts a 2% w/v aqueous solution of methylcellulose, Sigma Aldrich Product No. M0387 using a dip-in method.

B. The drops of methylcelluose semi-solid media as in **A**, shown in an almost vertically held Petri dish; the solution viscosity was 1,500 cPs.

Relatively high gelling temperature of 2% methyl cellulose, approximately 48 °C, prevents its use in a gelled state for 3D cell culture applications. While 10% methylcellulose solutions gel at approximately 30 °C, the solution viscosity is often too high for routine use. For this reason, methyl cellulose semi-solid media rather than a gel is used for the culture of human cells, clonal growth of cells, embryoid bodies, neurospheres etc, and further used in methylcellulose-based colony forming assays and anchorage independence assays. In addition to cell culture applications, cellulose ethers are used as excipients in drug formulations, or sustained release of other biomolecules. In solid tablets, cellulose ethers enable a swelling-driven release of the drug in contact with physiological fluids.

Methylcellulose is biodegradable (although not by animal and human cells in culture as they do not synthesize cellulases). The SeedEZ™ accepts methylcellulose semi-solid media as a 2% w/v aqueous solution (Fig. 7) at a viscosity of approximately 1500 cPs (Sigma Aldrich Product No. M0387).

SYNTHETIC GELS

PLURONIC

Concentrated aqueous solutions of poloxamers form thermo-reversible gels which revert to a liquid when the temperature is reduced. Poloxamers are triblock copolymers of a central hydrophobic chain of poly(propylene oxide) and two hydrophilic chains of (poly(ethylene oxide), PEO-PPO-PEO. Pluronic F127 is the trade name for nontoxic Poloxamer 407. The advantage of Pluronic F127 for 3D cell culture applications is that it forms a gel at physiological temperatures at concentrations higher than approximately 20% w/v. It is frequently used for cell encapsulation, as a substrate in tissue engineering (e.g. cartilage), as a component in drug delivery and pharmaceutical formulations, an additive to culture media in bioreactors, a surfactant and reagent which facilitates solubilization of hydrophobic molecules in water etc.

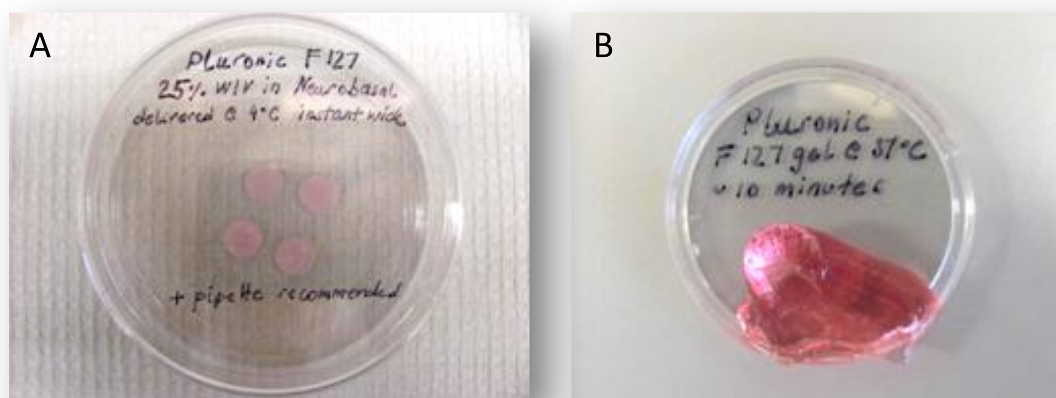

Fig. 8 Pluronic F127 (PEO-PPO-PEO) gel embedded in the SeedEZ.

A. Without pre-wetting treatments, the SeedEZ instantly wicks a 25% w/v Pluronic F127 solution in Neurobasal medium. The use of positive displacement pipette is recommended.

B. A sample of gelled Pluronic F127; the same as delivered to the SeedEZ substrates in **A**.

The making of aqueous poloxamer gels is simple and requires merely the addition of weighed amount of the poloxamer to cold water with slow mixing to prevent foaming. The gelling temperature depends on the poloxamer concentration, pH, and on the type and amount of additives, if any SeedEZ™ instantly wicks sol-state 25% w/v Pluronic F127 solution (Fig. 8). . Pluronic is biodegradable.

CARBOMER

Carbomer is a generic name for synthetic high molecular weight polymers of acrylic acid. These polymers have widespread use in pharmaceutical formulations for ocular, oral, transdermal and nasal drug delivery.

SeedEZ™ accepts Carbopol polymers by dip-in method (Fig. 9). Carbopol is the trade name for polymers of acrylic acid crosslinked with polyalkenyl ethers or divinyl glycol manufactured by Lubrizol Corporation. Carbopol 971P NF is used as the matrix ingredient for controlled release tablets and capsules, suspending agent for oral liquids, bioadhesive in drug delivery systems, etc. Lightly crosslinked polymers, such as Carbopol® 971P NF polymer tend to be more efficient in controlling drug release than highly crosslinked polymers such as Carbopol 974P NF polymer. Carbopol® 974P NF was introduced for use in oral and mucosal contact applications such as extended release tablets, oral liquids and bioadhesive formulations. It is a highly crosslinked polymer and produces highly viscous gels with rheology similar to mayonnaise.

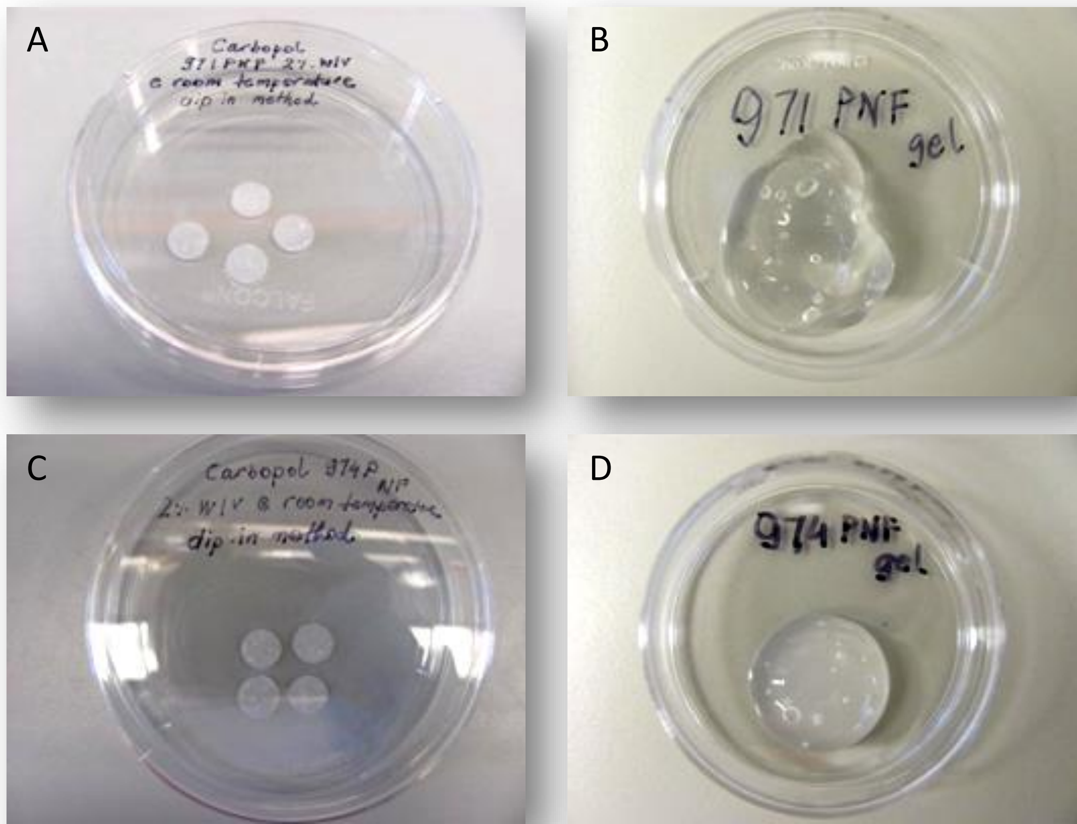

Fig. 9 Poly(acrylic acid) gel embedded in the SeedEZ.

A. Without pre-wetting treatments, the SeedEZ accepts Carbopol 971P NF, a 2% w/v solution in DI water using a dip-in seeding method.

B. A sample of gelled Carbopol 971P NF; the same as delivered to the SeedEZ substrates in **A**.

C. Without pre-wetting treatments, the SeedEZ accepts Carbopol 974P NF, a 2% w/v solution in DI water using a dip-in seeding method.

D. A sample of gelled Carbopol 974P NF; the same as delivered to the SeedEZ substrates in **C**.