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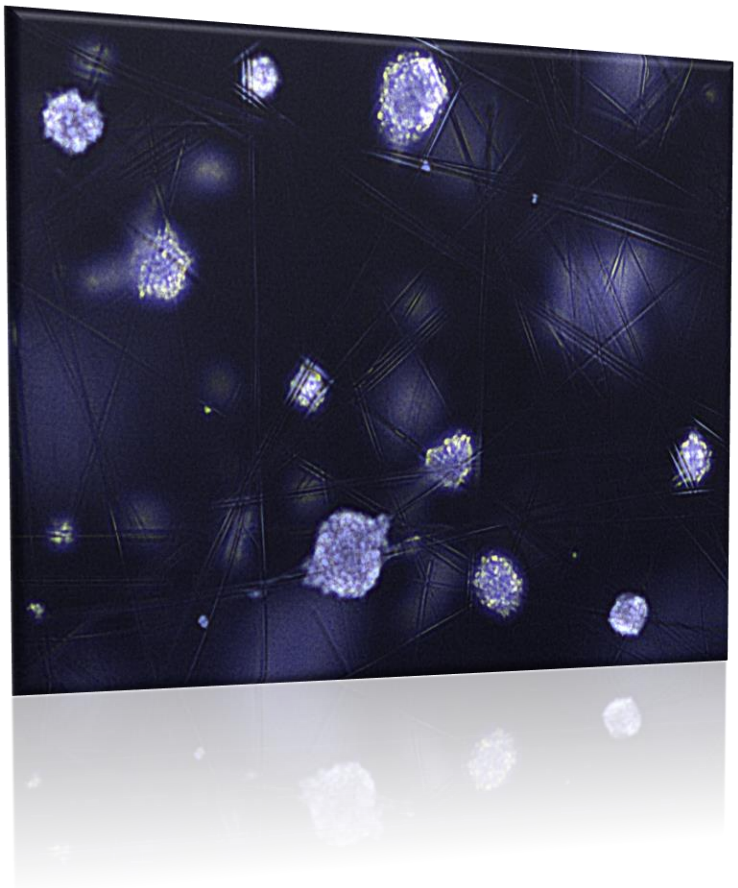
SEEDEZ™ PROTOCOLS

SEEDEZ™ COATINGS FOR SPHEROID CULTURES AND 3D CELL CULTURES OF SUBSTRATE-ADHERED CELLS



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SeedEZ™ Coatings for Spheroid Cultures and Substrate-Adhered 3D Cell Cultures

December 2018

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INTRODUCTION

Depending on cell types, application and research objectives, SeedEZ™ may be coated or uncoated. If coated, the SeedEZ™ may be coated with ligands which promote cell adhesion, or molecules which prevent cell adhesion to the SeedEZ™ substrate. The former provides a 3D network of round cells adhered to and spread inside the SeedEZ™. The latter provides for an aggregate 3D cell culture model or 3D cell spheroids cultured suspended within the interior of the SeedEZ™.

DESIRED 3D CULTURING CONDITIONS INFLUENCE SELECTION AND APPLICATION OF COATINGS

The SeedEZ™ is a unique substrate which allows cells to be cultured in three dimensions via predominantly:

1. Cell-to-SeedEZ™ substrate adhesion – 3D culture of cells adhered to coated SeedEZ™.
2. Cell-cell adhesion – 3D cell aggregates or spheroids suspended in the SeedEZ™.
3. Cell-cell and cell-extracellular matrix adhesion – 3D extracellular matrix gel cell culture embedded in the SeedEZ™.

However, these methods are not mutually exclusive. Depending on the method used you may need to or need not coat the SeedEZ™ with cell-adhesive molecules, or molecules which prevent cells from adhering to the SeedEZ™ substrate.

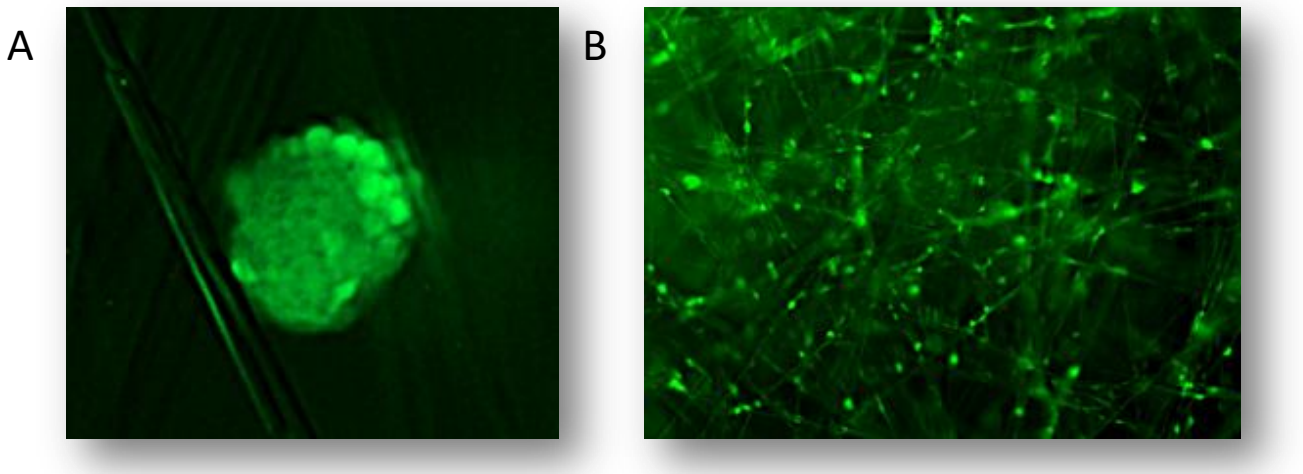

Fig. 1 The growth of neuronal and glial cells in **A** uncoated and **B** Poly-D-Lysine coated SeedEZ substrate.

A mixed population of dissociated cells comprising E-18 primary cortical neurons and P0-harvested and 1X passaged glial cells (astrocytes and microglia) was seeded into the SeedEZ substrates and cultured for 7 days in a serum-free medium. For these cell types, the SeedEZ by itself is not cell adhesive. On day 8, cells were stained using Calcein AM (live cells fluoresce green) and imaged using Nikon Eclipse 80i at 10X.

A. In the uncoated SeedEZ substrates there were very few cells. However, microscopic examination also revealed the presence of 3D cell clusters. Without cell adhesive molecules, dissociated cells at seeding formed 3D cell spheroids measuring approximately 50-150 µm in diameter which were suspended within the interior of the SeedEZ substrates. An image of the cell spheroid found in a 10 X field of view was cropped and enlarged to show the spheroid.

B. In the presence of Poly-D-lysine coating, dissociated cells adhered to the SeedEZ substrate and formed three-dimensional cell networks.

Analogous to planar disposables, surface treatment and coating versus no SeedEZ™ coating creates remarkable differences in culturing conditions and 3D cell organization (see Fig. 1). However, in contrast with anchorage-dependent cells of flattened morphology cultured in 2D, in the SeedEZ™, these differences influence growth and function of cells which are round and organized in 3D.

Fig. 1 shows a mixed 3D cell culture of neurons, astrocytes and microglia cultured in serum-free medium for 7 days in an uncoated SeedEZ™ substrate (Fig. 1A) and Poly-D-Lysine (PDL) coated SeedEZ™ substrate (Fig. 1B). As can be seen in Fig. 1A without a suitable coating, dissociated neural and glial cells lacked cell adhesive molecules and clumped, spontaneously forming 3D cell culture aggregates or spheroids in the interior of the SeedEZ™. Without necessary substrate-to-cell adhesion, cells adhered to each other to survive. When the appropriate molecule was coated, cells adhered and spread within the interior of the SeedEZ™ substrate as shown in Fig. 1B.

COATING RECOMMENDATIONS FOR DIFFERENT 3D CELL CULTURE MODELS

3D CULTURE OF ANCHORAGE-DEPENDENT CELLS ADHERED TO SEEDEZ™

When cell-to-SeedEZ™-substrate adhesion is the objective, please proceed with use of cell adhesive molecules and coating protocols normally used to improve cell adhesion, spread, and growth on standard tissue culture treated polystyrene and glass substrates. In general, when anchorage-dependent cells cultured on planar (2D) substrates need cell adhesive ligands for consistent adhesion, growth and spread, they need the same ligands when cultured in 3D, adhered to the SeedEZ™.

3D CELL SPHEROIDS SUSPENDED IN THE INTERIOR OF THE SEEDEZ™

When 3D cell spheroid formation and spheroid culturing is the objective, you may either seed dissociated cells to aggregate and form spheroids in the interior of the SeedEZ™ or seed pre-formed spheroids for suspension culture in the SeedEZ™. In both cases SeedEZ™ may be coated or uncoated depending on cell types and application.

To form 3D cell spheroids from a suspension of dissociated cells, try uncoated SeedEZ™ substrate first. Next, one hour after seeding, inspect the bottom of the well in which the SeedEZ™ was seated using an inverted microscope. If many cells settled and passed through the SeedEZ™, you may:

A. Seed dissociated cells in a SeedEZ™-STACK, a stack of two or more overlaid *uncoated* SeedEZ™ substrates.

B. Seed cells into a *coated* SeedEZ™.

   Use this approach to seed cells which tend to aggregate even in the presence of such coating on a planar surface.

C. Seed cells in a low-concentration *non-gelling* sol-sate hydrogel into an *uncoated* SeedEZ™.

D. Seed cells in a low-concentration *non-gelling* sol-sate hydrogel into a *coated* SeedEZ™.

E. Seed cells in a *gelling* sol-sate hydrogel into an *uncoated* SeedEZ™.

F. Seed cells in a *gelling* sol-sate hydrogel into a *coated* SeedEZ™.

Without appropriate coatings and/or sera some cells may pass through the SeedEZ™ if they could not adhere. The above methods allow cells which normally aggregate to aggregate within the interior of the SeedEZ™ rather than "pass" through it. Cells which aggregate in a flat dish and do not spread in a coated dish (or in a hydrogel) for a period of time in culture do the same in the SeedEZ™ under these conditions. This is because SeedEZ™ is 3D substrate with fewer points to contact cells than in a 2D culture. This helps spheroids form and grow in a "suspended" state in its interior. For many applications, coatings or hydrogels such as alginate,

agarose or methylcellulose prevent cells from "passing" through the substrate while allowing spheroids to form. Still, depending on cell type, other animal-derived or synthetic molecules may also be used.

Pre-formed cell spheroids may also be seeded into the SeedEZ™ for a suspension-like culture in the SeedEZ™ interior. Whether an additional coating is needed to prevent cells from adhering to the SeedEZ™ depends on cell types, culturing conditions such as presence of sera and other factors.

3D CULTURES OF CELLS EMBEDDED IN AN EXTRACELLULAR MATRIX GEL IN THE SEEDEZ™

When cell-cell and cell-extracellular matrix adhesion is the objective, it is recommended that dissociated cultures be seeded in an extracellular matrix sol-state suspension into the SeedEZ™. This is critical for those cell types which do not form their endogenous matrix support or do so at later time points in culture, yet they need these molecules to survive.

For most applications, when cells are seeded in a sol-state ECM gel into the SeedEZ™, no additional pre-coating of the SeedEZ™ is necessary. However, if you commonly pre-coat disposables before plating cells in a gel, continue with your protocols.

COATINGS FOR SPHEROID CULTURES SUSPENDED IN THE SEEDEZ™

3D cell spheroids are formed by culturing principles and tools which promote cell-to-cell adhesion. These methods typically deprive cells from significant substrate-to-cell adhesion and/or provide for culturing geometry in which cells cluster and aggregate to form a spheroid. Examples include tumor spheroids, stem cell spheroids and other tissue models used in diverse applications ranging from the *in vitro* cell therapies, to studying organogenesis, to screening.

YOU MAY SEED DISSOCIATED CELLS WITH THE OBJECTIVE TO FORM 3D CELL SPHEROIDS IN THE SEEDEZ™, OR YOU MAY SUSPEND SMALLER OR LARGER PRE-FORMED 3D CELL AGGREGATES AND/OR SPHEROIDS INTO THE SEEDEZ™.

To prevent cells or formed spheroids from adhering to the SeedEZ™ substrate, test the uncoated SeedEZ™ first.

While the SeedEZ™ may be more cell adhesive than an ultra-low attachment surface, it is a 3D substrate, approximately 90% void, and with a fewer "points" to contact cells than a 2D (planar) substrate. If you find that cells or aggregates stick to the SeedEZ™ and spread, proceed with the SeedEZ™ coating using non-cell-adhesive molecules you normally use.

SPHEROIDS FORMING FROM A SEEDED SUSPENSION OF DISSOCIATED CELLS IN MEDIUM

To form spheroids from a single cell suspension in medium, you may seed cells into *uncoated* or *coated* SeedEZ™ substrates placed into wells of untreated or low-attachment-treated polystyrene plates.

In a stationary culture, an uncoated substrate is often not retentive enough to prevent some cells from passing through. To prevent this, use a SeedEZ™-STACK, two or more overlaid uncoated SeedEZ™ substrates to confine all cells. You may also coat the SeedEZ™ with "sticky" molecules to prevent cells from passing through the substrate and use just one SeedEZ™ substrate. An agarose, alginate, methylcellulose or other coating may be used as a "sticky" coating. If cell types used form spheroids easily, you may even coat the

SeedEZ™ with extracellular matrix molecules to confine all cells to the SeedEZ™ interior and the cells will still form spheroids.

For some cell types, reducing serum concentration during the initial days in culture may make cells cluster, aggregate and form spheroids. In addition to stationary cultures, you may also culture dissociated cells in the SeedEZ™ in a multi-well plate seated on an orbital shaker as this motion helps cells aggregate and form spheroids.

Cell density also plays a role. When seeded into the SeedEZ™, cells aggregate easier when there are many cells. Spheroid formation is also facilitated by the seeding of dissociated cells in a sol-state hydrogel, where the hydrogel subsequently gels or does not gel, as described in the next section. For those cells types that typically cluster and aggregate, cell dissociation which does not yield a *single cell* suspension but rather a suspension of small aggregates comprising few cells, generally forms spheroids faster and/or easier than does a single cell.

In general, the decision on whether to coat the substrate or not depends on the cell type, whether culturing conditions are stationary or not, and most importantly on your previous experience with spheroid forming cell types.

SPHEROIDS FORMING FROM A SEEDED SUSPENSION OF DISSOCIATED CELLS IN A SOL-HYDROGEL

You may seed cells in undiluted or diluted sol-state gel suspension into the SeedEZ™. *For many applications, no additional coating is necessary under these conditions*. At low concentrations hydrogels do not gel but form viscous cell suspension which "sticks" to the interior of the SeedEZ™; thus, retaining cells. At higher concentrations, hydrogels gel. A gelled hydrogel may restrict subsequent spheroid growth. The SeedEZ™ solves this problem and allows cell seeding in a low concentration hydrogel as it is cell retentive itself. This is advantageous because at a low concentration, most gels do not form stable gels and degrade. In turn, gel degradation provides for less constrained spheroid growth. Finally, spheroids remain suspended inside 400 µm thick SeedEZ™ even when the gel is degraded and can no longer support cells or spheroids in 3D.

Often used hydrogels for this application are alginates and agarose. However, other gels and extracellular matrices such as Matrigel and Collagen are also used depending on cell type in question. For more information please see the SeedEZ™ Gel Embedding Application Note.

SPHEROIDS FORMING FROM A CELL SUSPENSION IN MEDIUM SEEDED ONTO A GEL IN THE SEEDEZ™

You may also seed cells on top of the dry, semi-dry, or hydrated hydrogel embedded in the SeedEZ™ provided that the gel is permeable to cells. For more uniform cell seeding into the interior of the gel embedded in the SeedEZ™, centrifuge the plate comprising SeedEZ™ substrates with the embedded gel. *For many applications, no additional coating is necessary under these conditions*. However, whenever a hydrogel is used, consider the needs of the cell type in question in choosing a suitable hydrogel and its concentration. In general, different cell types have different needs with respect to matrix environment.

PRE-FORMED SPHEROIDS SEEDED INTO AND SUSPENDED IN THE SEEDEZ™

Cell spheroids formed in conical bottom plates, AggreWell plates, rotary suspension culture or other methods may also be seeded into the SeedEZ™. Depending on cell types and spheroid size, you may need not coat SeedEZ™ with molecules that prevent cell-to-substrate adhesion. It is recommended that spheroids be seeded into uncoated SeedEZ™ substrates first. If they stick to the SeedEZ™ and cells spread, and the objective is to maintain cells in a spheroid, proceed with SeedEZ™ coating using protocols you normally use to prevent cell-to-cell ware adhesion.

For culturing spheroids in a suspension state in the SeedEZ™, place the SeedEZ™ into a hydrophobic dish or plate; for example, in the wells of untreated- or low-attachment multi-well plate.

SUGGESTED SEEDEZ™ COATINGS AND MULTIWELL PLATE SURFACES FOR SPHEROID CULTURES

For culturing spheroids in the SeedEZ™, it is recommended that the SeedEZ™ be placed in untreated polystyrene, Nunc low-cell binding surface- or Corning ultra-low attachment surface disposables. These surfaces prevent cell adhesion.

If cells stick to the SeedEZ™, proceed with the SeedEZ™ coating using a cytophobic solution you commonly use; for example, agar, agarose, alginate, methylcellulose etc. or email support@lenabio.com for a coating recommendation. You may use these molecules as a thin-coat or as a gelled gel embedded in the SeedEZ™ (see SeedEZ™ Gel Embedding Application Note).

COATINGS FOR 3D CELL CULTURES IN AN EXTRACELLULAR MATRIX GEL IN THE SEEDEZ™

If the objective is to seed anchorage-dependent cells in a sol-state extracellular matrix (ECM) suspension into the SeedEZ™, and you previously formed 3D gel cell cultures by coating the dishes to provide for good gel-to-dish adhesion, continue with protocols and coat the SeedEZ™ followed by seeding cells in a sol-state ECM.

You may find that this step is not necessary because the gelled ECM provides for good culture adhesion to the interior of the SeedEZ™ substrate, even when your 3D gel cell cultures "peels off" if plated onto a flat surface.

COATINGS FOR ADHESION OF ANCHORAGE-DEPENDENT CELLS TO THE SEEDEZ™

Anchorage-dependent cells need adhesion to substrates to survive. Tissue culture treated polystyrene dishes, flasks, and multiwell plates may not be cell adhesive enough for all cell types to provide for consistent culture growth without sera and cell specific medium supplements.

Anchorage-dependent cells cultured in low- or serum-free media need cell-adhesive molecules (coatings) for adequate cell attachment and spreading to the substrate on which they are cultured. These molecules are cell-specific; however, most coatings will meet adhesion requirements of more than one cell type. Once the coating solution is prepared and aliquoted, the process of coating multi-well plates and other cell ware disposables is easy to do and automate as it comprises merely the steps of additions, incubations and aspirations. As with any other cell ware disposable, you may easily apply this process to the SeedEZ™.

GUIDELINES AND RECOMMENDATIONS

SeedEZ™ is hydrophilic and absorbent; however, just like any other disposable it may not be cell-adhesive enough for all cell types. Fig. 1 shows that mixed 3D cell culture of neural and glial cells cultured in serum-free medium required a suitable coating for good adhesion and spread within the interior of the SeedEZ™ substrate. For these cell types, users are instructed to coat the SeedEZ™, unless the objective is to form aggregates.

Cell culture disposables are coated to improve cell attachment, growth and proliferation. If you normally coat disposables, continue with your protocols and coat the SeedEZ™. Most coatings used with standard tissue

culture treated polystyrene or glass, may be readily applied to the SeedEZ™ without any additional pre-treatments.

The following guidelines may assist in optimizing or troubleshooting your current coating protocols:

A. Cells vary in their requirements for adhesion in terms of substrate stiffness, charge, and wettability.

    a. In some instances, poor culture growth and survival cannot be attributed solely to the poor selection of the coating and its performance with cells in question during culture. The substrate stiffness may play a role and be equally or more important.

    b. Cells may be able to grow on surfaces that are either positively or negatively charged. In some cases, the charge density may be more important than the surface polarity.

B. Not all coatings are equally stable in culture and not all coatings "stick" sufficiently to the substrate.

C. Coatings may degrade. Degradation is typically hydrolytic or enzymatic whether by cell activity, presence of certain constituents in cell culture reagents, handling, or due to environmental factors. In some instances, you may be able to obtain a more stable coating by:

    a. Using a higher polymer concentration in the coating solution.

    b. Applying longer period of substrate incubation in the coating solution.

    c. Using a different molecular weight of the polymer.

    d. Using a slightly different polymer composition.

    e. By protecting plates from certain environmental factors.

    f. Using a combination of polymers.

D. Untreated polystyrene surfaces are hydrophobic and not suitable for culture of most anchorage-dependent cells.

    a. These surfaces are used in bacterial dishes, and disposables for suspension cultures or cell spheroids.

    b. While gamma sterilization improves wetting and somewhat increases binding on untreated polystyrene, this is still insufficient for even spread and consistent growth of many anchorage-dependent cell types.

    c. Even with sera, the adhesion of anchorage-dependent cells to untreated polystyrene may still be poor.

    d. Corona or gas plasma treatment of the untreated polystyrene may be necessary for sufficient wetting or binding of the coating solution to the surface. In this case, tissue culture treated polystyrene, i.e. corona treated plate or flask, may save you time and effort.

E. Hydrophilic surfaces are generally more suitable for growth of adherent cells; however, not all hydrophilic coatings are cell adhesive.

COMMON COATINGS

COATINGS COMPRISING EXTRACELLULAR MATRIX CONSTITUENTS

COLLAGEN I

Collagen is the main component of connective tissues and the most abundant protein in the extracellular matrix. Collagen Type I provides structural support to cells and facilitates attachment, growth, differentiation and migration. Among cells grown 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 and PC12.

Collagen I can be applied as a coating or as a gelled matrix to standard cell culture disposables and the SeedEZ™. SeedEZ™ instantaneously wicks gelling bovine Type I Collagen as a sol-state gel at a protein concentration of 2.9 mg/ml. For more information please see SeedEZ™ Gel Embedding Protocols.

COLLAGEN IV

Collagen Type IV is present in basement membranes. It can be applied as a coating or as a gelled matrix to standard cell culture disposables and the SeedEZ™. Examples of cells which show enhanced attachment and growth on Collagen IV are epithelial cells, human epidermal stem cells, keratinocytes, mouse ES cells, and HT-1080 cells.

LAMININ

Laminin is the major glycoprotein of basement membranes. It has been shown to stimulate neurite outgrowth and promote cell attachment, differentiation, chemotaxis and neuronal survival.

It can be applied as a coating at 1-2 µg/cm^2 (Sigma-Aldrich Product No. L2020) to cell culture disposables and the SeedEZ™ or used as a gelled matrix at a higher protein concentration. SeedEZ™ accepts Laminin and instantly wicks undiluted (L2020) Laminin stock solution (Fig. 2). Laminin has been used for culture of neuronal cells, keratinocytes, myoblasts, and breast epithelial cells.

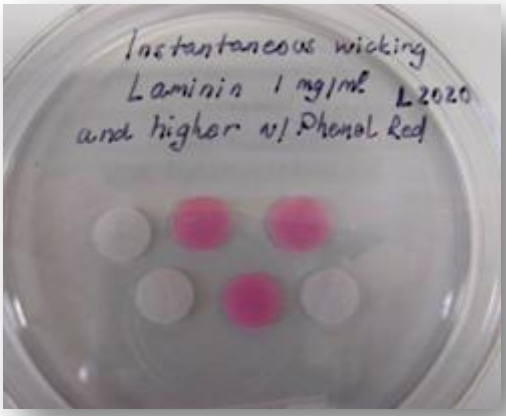

Fig. 2 Laminin-coated SeedEZ.

Without pre-wetting treatments, the SeedEZ instantly wicks undiluted Laminin, Sigma–Aldrich Product No. L2020, at a protein concentration of 1 mg/ml and a 1.5 mg/ml Laminin with Phenol Red diluted from stock.

MATRIGEL

Matrigel is a gelatinous protein mixture secreted by EHS murine sarcoma and marketed by BD Biosciences, and by Trevigen under the name Cultrex BME, Life Technologies under the trade name Geltrex, Sigma-Aldrich under the name ECM Gel or the EHS matrix. The main components of Matrigel are structural proteins Laminin, Collagen IV and Entactin. According to manufacturer, Matrigel provides a physiologically relevant surface for a range of 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.

SeedEZ™ accepts Matrigel either as a coating or as a sol-state gel up to 16 mg/ml protein concentration (the highest protein concentration tested; solution obtained from stock of Growth Factor Reduced Matrigel, High Concentration; BD Biosciences Product No. 354263). For more information please see the SeedEZ™ Gel Embedding Protocols.

FRIBRONECTIN

Fibronectin is a large, cell surface and plasma protein with structural and adhesive properties in cell-associated fibrillar matrices. It is one of the primary cell adhesion molecules.

The principal function of human Fibronectin appears to be in cell migration during wound healing and development, regulation of cell growth and differentiation, and haemostasis. Among cell types which attach, spread, and proliferate on Fibronectin-coated cultureware are smooth muscle cells, endothelial cells, mesenchymal cells, neuroblastoma cells, neuronal cells, neural crest cells, human myeloma cell lines and fibroblasts.

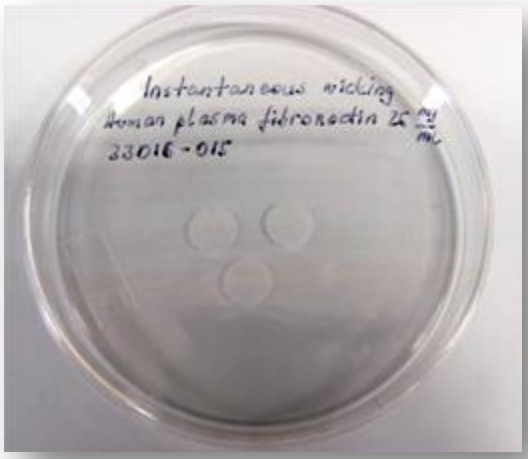

Fig. 3 HFN-coated SeedEZ.

Without pre-wetting treatments, the SeedEZ instantly wicks Human Plasma Fibronectin, Life Technologies Product No. 33016-015, 2.5 mg/ml solution.

SeedEZ™ accepts Fibronectin (see Fig. 3). MC3T3-E1 pre-osteoblastic cells were successfully cultured in 3D adhered to and embedded within 20 µg/ml bovine plasma Fibronectin-coated SeedEZ™ substrates (Sigma-Aldrich Product No. F1141).

COATINGS DERIVED FROM NATURAL SOURCES

GELATIN

Gelatin is a mixture of water-soluble peptides and proteins produced by partial hydrolysis of Collagen. It is also a thermo-reversible hydrogel which stiffness depends on gelatin composition, concentration in solution, pH and temperature. Fig. 4 shows Type A (derived from acid-cured tissue) and Type B gelatin (derived from

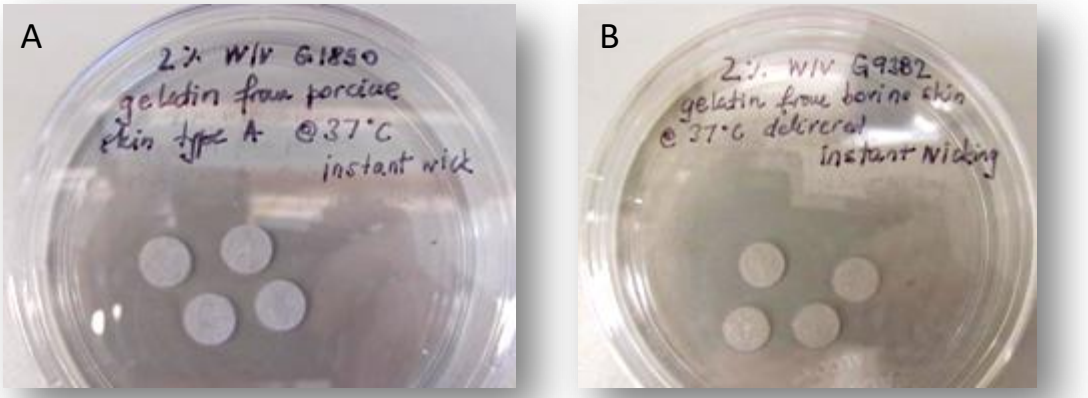

Fig. 4 Gelatin-coated SeedEZ (2% w/v solution in DI water cooled down and delivered to SeedEZ substrates at 37°C).

Without pre-wetting treatments, the SeedEZ instantly wicks:

A Type A gelatin from porcine skin (Sigma-Aldrich Product No. G1890).

B Type B gelatin from bovine skin (Sigma-Aldrich Product No. G9382).

lime-cured tissue) as a 2% w/v aqueous solution, cooled to 37°C and delivered to the SeedEZ™ in sol-state. According to supplier, at this concentration gelatin is suitable for use as a cell culture substratum by coating disposables at 5-10 µl/cm^2. A 2% w/v gelatin solution coated surfaces should be allowed at least 2 hours to

dry before introducing cells and medium. Optimal concentration depends on cell type, application and the research objectives.

Gelatin provides an attachment and growth promoting substrate for the culture of many cell types. Vascular endothelial cells, HUVEC cells, and embryonic stem cells have been successfully cultured on gelatin. The SeedEZ™ accepts gelatin either as a coating or as a sol-state gel.

NON-ANIMAL ORIGIN DERIVED COATINGS

Agarose, methylcellulose, and alginate are the examples of cell culture substrata derived from cellulose or multicellular algae. The SeedEZ™ accepts agarose, methylcellulose and alginate either as a coating or as a sol-state hydrogel. For more information please see the SeedEZ™ Gel Embedding Protocols.

SYNTHESIZED POLYMER COATINGS

POLY-L-LYSINE AND POLY-D-LYSINE

Tissue culture treated polystyrene surface is hydrophilic and negatively charged. Poly-Lysine (PL) polymers enhance cell adhesion and protein absorption by altering the surface charges. They are available in L and D conformations. Poly-L-Lysine (PLL) polymers can be degraded and digested by some cell types. In that case, Poly-D-Lysine (PDL) polymers should be used as they are stable. PDL forms a uniform net positive charge on the plastic surface which, for some cell types, enhances cell attachment, growth and differentiation in serum-free or low serum media, and reduces cell detachment or culture peeling from polystyrene surfaces during routine media exchanges and washing steps in cell-based assays.

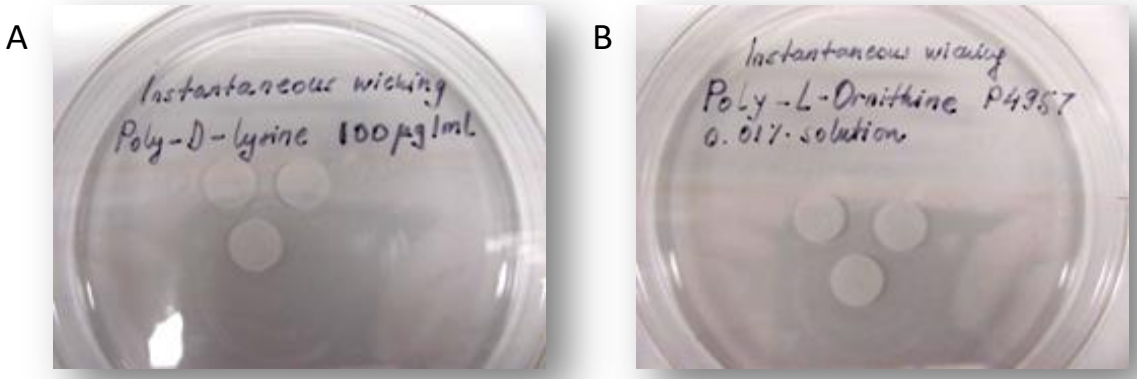

Fig. 5 Poly-D-Lysine and Poly-L-Ornithine coated SeedEZ.

Without pre-wetting treatments, the SeedEZ wicks:

A Poly-D-Lysine 100 µg/ml solution in sterile DI water (Sigma-Aldrich Product No. P7405).

For PDL MW < 300 kDa, 0.01% w/v solution of PDL is wicked instantly. For PDL MW > 300 KDa, 0.01% w/v solution of PDL is wicked instantly or within 1-5 seconds.

B Poly-L-Ornithine 100 µg/ml solution in sterile DI water (Sigma-Aldrich Product No. P4957).

At a molecular weight of 30-70 kDa, a ready to use Poly-L-Ornithine solution was wicked instantly.

Poly-Lysine polymers with a molecular weight of 70 - 300 kDa are used to provide for adequate cell adhesion. However, the higher the molecular weight, the more viscous the coating solution becomes and more difficult to work with. Polymer selection, whether PDL or PLL, and molecular weight, should account for the susceptibility of the polymer to cell digestion, required attachment site density, and viscosity with accompanied difficulty in handling.

PDL and PLL are synthetic compounds. They do not stimulate biological activity in resident cells. Many cell types will adhere to Poly-Lysine. Examples of cell types cultured on PL- treated surfaces include primary neurons, glial cells, neuroblastomas, neuronal cells lines, and many transfected cells lines. SeedEZ™ accepts both Poly-L-Lysine and Poly-D-Lysine, even as viscous, relatively high molecular weight polymers (Fig. 5A). The wicking is typically instantaneous; however, it may take up to 10 seconds or longer for highly viscous solutions to be wicked into the interior of the SeedEZ™.

POLY-L-ORNITHINE

Poly-L-Ornithine is yet another positively charged synthetic amino acid polymer chain used as a coating to enhance cell attachment and adhesion to both plastic and glass surfaces. Examples of cells cultured on Poly-L-Ornithine treated surfaces are cells of neuronal and glial lineage. The SeedEZ™ accepts Poly-L-Ornithine coating (Fig. 5B).

As coatings, PDL, PLL and Poly-L-Ornithine are exogeneous source of attachment factors for cells cultured in low or serum-free media and for cells which do not synthesize attachment factors and extracellular matrix constituents.

PROTOCOL: 3D culture of osteoblastic cells in uncoated, PDL- and Fibronectin-coated SeedEZ™

The protocol describes how to coat the SeedEZ™ substrates for the culture of MC3T3-E1 osteoblastic cells adhered to and embedded within uncoated, Fibronectin-coated, and Poly-D-Lysine-coated SeedEZ™ SC-C048 substrates in a 48-well plate. Cells were seeded at approximately 150,000 live cells per well.

FIBRONECTIN COATING

MATERIALS

- Fibronectin from bovine plasma; Sigma-Aldrich F1141, a 1 mg/ml sterile-filtered bioreagent suitable for cell culture.
- Sterile, balanced salt solution
- Disposables: SeedEZ™ substrates SC-C048, a 48-well plate, conical tubes, and micropipette tips

METHODS

1. Prepare 20 µg/ml Fibronectin in a sterile balanced salt solution. Store the coating solution at 2-8 °C.
2. Place SC-C048 SeedEZ™ substrates into a 48-well plate inside the hood.
3. Add 250 µl of 20 µg/ml Fibronectin solution to each well comprising SeedEZ™.
4. Allow to air dry for at least 45 minutes. Excess Fibronectin should be removed by aspiration.
5. Rinse the substrates 2X with sterile DI water.
6. After you aspirate the last rinse, dry the SeedEZ™ substrate by placing the pipette tip on the substrate and aspirating any remaining liquid from the SeedEZ™. You will notice the color change from transparent to white when dried.
7. Cover the plate. Fibronectin-coated SeedEZ™ substrates are ready for use.

POLY-D-LYSINE COATING

MATERIALS

- Poly-D-lysine Hydrobromide MW >300 kDa lyophilized powder, γ-irradiated; Sigma-Aldrich Product No. P7405
- Sterile, de-ionized water
- Disposables: SeedEZ™ substrates SC-C048, a 48-well plate, conical tubes, and micropipette tips

METHODS

1. Prepare 100 µg/ml Poly-D-Lysine coating solution in sterile DI water. Store at 2-8 °C.
2. Place SC-C048 SeedEZ™ substrates into a 48-well plate inside the hood.
3. Add 250 µl of 100 µg/ml Poly-D-Lysine solution to each well comprising SeedEZ™.

4. Cover the plate by lid and transfer to incubator for at least 6 hours. Overnight incubation recommended.
5. Transfer the plate to hood and aspirate PDL.
6. Rinse the substrates 2X with sterile DI water.
7. After you aspirate the last rinse, dry the SeedEZ™ substrate by placing the pipette tip on the substrate and aspirating any remaining liquid from the SeedEZ™. You will notice the color change from transparent to white when dried.
8. Cover the plate. PDL-coated SeedEZ™ substrates are ready for use.

MAINTENANCE AND DISSOCIATION OF MC3T3-E1 CELLS PRIOR TO SEEDING

MATERIALS

- MC3T3-E1 cells
- Medium: Minimum Essential Medium (MEM) α-Medium + 10% FBS + 1% antibiotic/antimycotic
- Phosphate-Buffered Saline (PBS) 1X
- 0.25% Trypsin - EDTA
- HBSS, Trypan Blue and hemocytomer for cell counting
- Disposables: T-75 flasks, conical tubes, serological pipettes, and micropipette tips

METHODS

1. Maintain cells in T-75 flasks in 10 ml of medium.
2. Passage cells according to supplier protocol.
3. When approximately 80% confluent, dissociate for seeding into the SeedEZ™ as follows:
4. Transfer 0.25% Trypsin - EDTA and medium to a 37°C water bath to warm up.
5. Aspirate medium from the flask.
6. Add 10 ml of 1X PBS to the flask and gently rinse the cells. Aspirate PBS.
7. Add 3 ml of 0.25% Trypsin-EDTA (1X) and gently tilt the flask to ensure that the entire bottom surface is covered.
8. Transfer flask to incubator for 1-2 minutes.
9. Observe cells under the microscope:

   If most cells are detached, transfer the flask to hood.

   If cells are still adhered, place the flask back to incubator for 1 minute; repeat this step until cells are detached.
10. Add 7 ml of medium to the flask to neutralize Trypsin.

11. Use 10 ml serological pipette and rinse the bottom of the flask with medium/Trypsin mixture repeatedly to collect as many cells as possible.

12. Transfer cell suspension to a 50 ml conical tube and spin at 1000 RCF for 5 minutes (1000 RCF = 1000 x g).

13. Remove supernatant and re-suspend cell pellet in 1-2 ml of medium.

14. Count cells using hemocytometer and Trypan Blue exclusion.

15. Seed into the SeedEZ™ at a desired cell density.

1-WEEK CULTURING RESULTS

MC3T3-E1 cells were cultured in uncoated, Fibronectin-coated, and Poly-D-Lysine-coated SeedEZ™ SC-C048 substrates (4 substrates per condition) in a 5% CO_2 37°C incubator. On day 8, cells were stained using Calcein AM (live cells green) and Hoechst 33342 (all cell nuclei blue). Cells were imaged at 4X. The results are shown in Figs. 6-8.

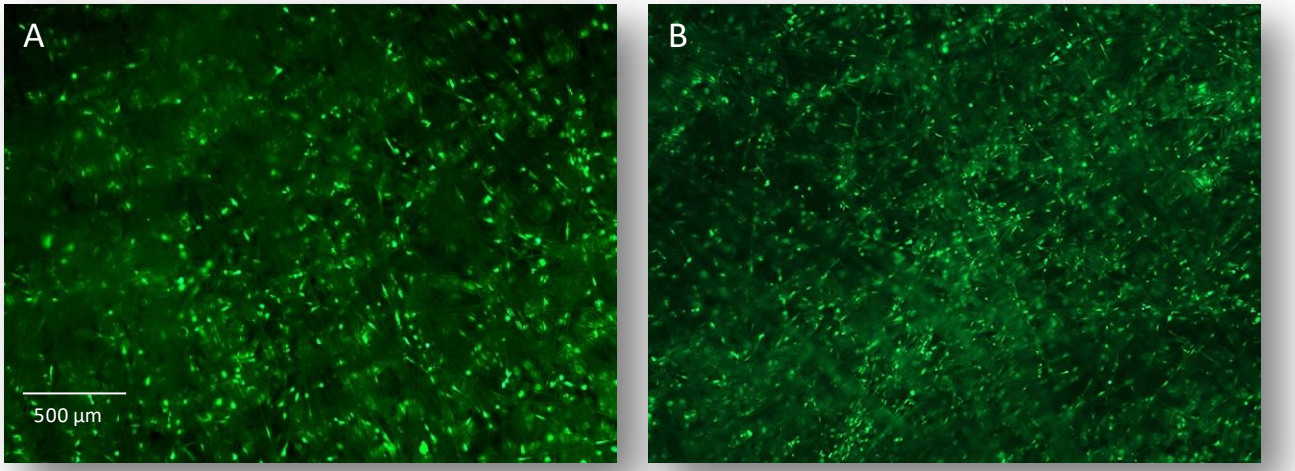

Fig. 6 MC3T3-E1 osteoblastic cells cultured for 1 week in the SeedEZ substrates.

A. Cells cultured in 20 µg/ml Fibronectin-coated SeedEZ substrate.

B. Cell cultured in uncoated SeedEZ substrate.

Cells were seeded at 150,000 cells/substrate. The medium was α-MEM + 10% FBS + 1% antibiotic/ antimycotic and was replaced every 2-3 days in culture. On Day 8, cell were stained using Calcein AM (live cells green) and Hoechst 33342 nuclear counterstain (all nuclei blue) and imaged on Nikon Eclipse 80i at 4X. Calcein AM staining is shown.

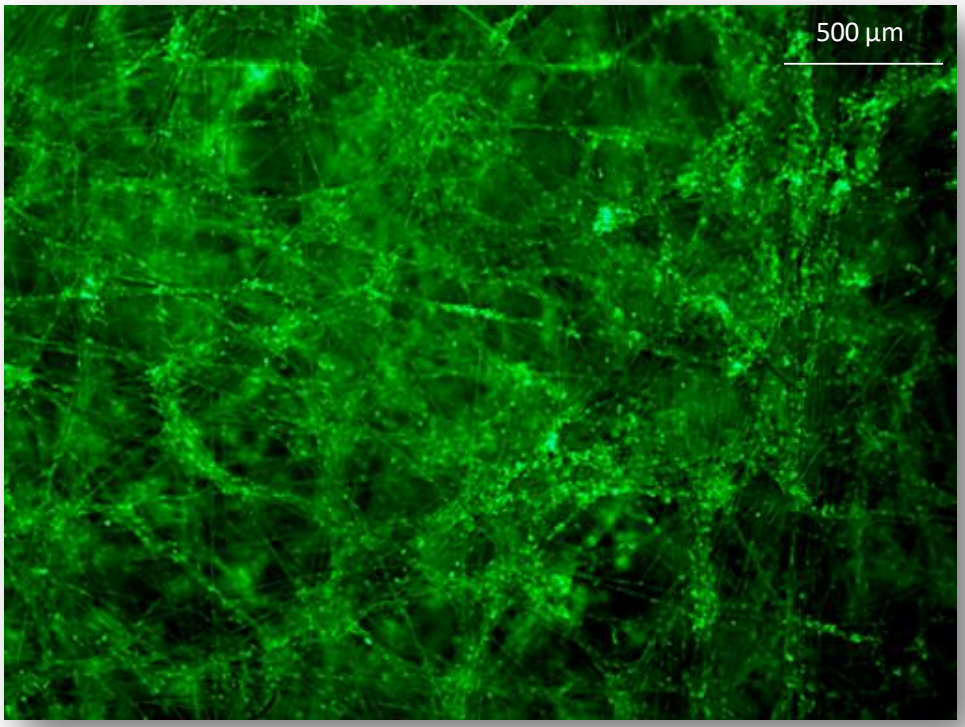

Fig. 7 Calcein AM stained MC3T3-E1 osteoblastic cells cultured for 1 week in PDL-coated SeedEZ substrates.

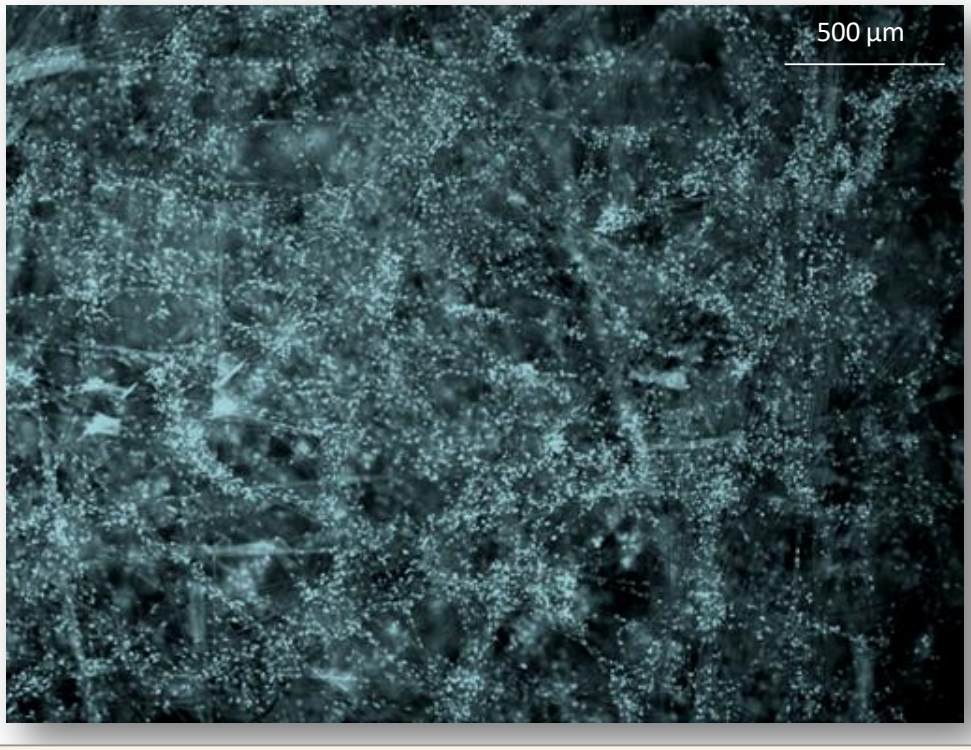

Fig. 8 Hoechst 33342 stained MC3T3-E1 osteoblastic cells cultured for 1 week in PDL-coated SeedEZ substrates.

RESULTS SHOW THE FOLLOWING:

I. Cells formed 3D cell networks and thrived in *uncoated*, *PDL-coated* and *Fibronectin-coated* SeedEZ™ substrates.

II. SeedEZ™ is transparent.

III. Cells can be imaged in the interior of *uncoated* and *coated SeedEZ™* using a standard fluorescence microscope.

IV. The cultures were indeed three-dimensional. First, the top of the culture was imaged. Next, the SeedEZ™ comprising 3D cell culture was flipped and the opposite side of the culture was imaged. Both sides of the culture were imaged and had live cells. This was true for all 12 cultures.

V. After 1 week in culture (and up to 8 weeks in culture; data not shown), the SeedEZ™ remains stable and rigid enough that a 3D cell culture embedded in the SeedEZ™ can be handled using sterile tweezers. For example, you may easily flip the substrate to image from both sides to resolve cell distribution through the culture thickness or transfer the culture to another dish or any other cell ware disposable.