

Using Polymers to Build Three Dimensional Coculture Systems for Angiogenesis

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Abstract Combining stem cells with polymers allows one to build new microenvironments and to investigate the role surface molecules, mechanical properties, and soluble factors play on stem cells in a physiologically relevant three dimensional system. In this chapter, we will focus primarily on commercially available polymers and those that can be made very simply with a limited knowledge of chemistry. We will also cover simple techniques to process the polymers to create architectures that can direct cell interactions.

1 Introduction

The concept for combining polymer scaffolds or substrates with cells is as the basis of the field of tissue engineering. It was observed that the introduction of cells alone often could not recapitulate large defects. However, when a polymer scaffold or substrate was combined, some cells have been able to recapitulate tissues [1]. As the field has developed, researchers have come to realize that the polymer scaffolds can be used to recapitulate microenvironments to direct the survival and differentiation of cells including stem cells [2–4].

Polymer scaffolds have become more than a temporary structure. They are being used as tools to engineer the molecular interactions between cells and their environment. They can be used to investigate the molecular cues that influence cell fate and tissue development. By choosing the appropriate polymer and process one can control the mechanical properties, the surface interactions, and the soluble factors that interact with the stem cells. This approach holds tremendous promise for new insight into matrix biology, but to capitalize on this, one must have a basic understanding of the polymers that may act as the foundation for these microenvironments.

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In this chapter, we will focus primarily on commercially available polymers and those that can be made very simply with a limited knowledge of chemistry. We will also cover simple techniques to process the polymers to create architectures that can direct cell interactions.

1.1 The Degradable Polyesters

Some of the most commonly used polymers for building scaffolds and designing microenvironments for stem cells are based on degradable polyesters. These polymers are commercially available, and they can be processed into a wide range of structures. Furthermore, by choosing the molecular weight of the polymer, and, in the case of copolymers, the ratio of subunits, one can tailor how stiff the material is, and how fast it degrades.

1.1.1 PLA, PGA, and PLGA

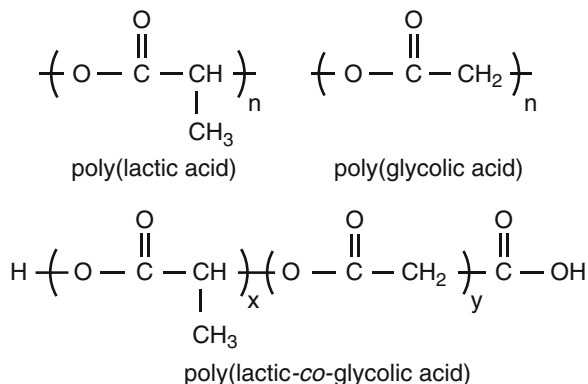
Poly(lactic acid) (PLA), poly(glycolic acid) (PGA), and their copolymer, poly(lactic-*co*-glycolic acid) (PLGA), have been employed in a number of devices approved for use by the FDA [5, 6]. They are sometimes called polyglycolide and polylactide in reference to their monomers. They degrade by hydrolysis wherein water cleaves the ester linkages. While they can degrade by either base hydrolysis (catalyzed by base) or acid hydrolysis, catalyzed by acid, in the body, they degrade by acid hydrolysis to lactic and glycolic acids, which are excreted. The degradation rate is controlled by the molecular weight of the polymer, the crystallinity, and the ratio of glycolic acid to lactic acid subunits. Since lactic acid is more hydrophobic than glycolic acid due to the methyl group, hydrolysis is slower for PLA than PGA of the same molecular weight and degree of crystallinity.

PLA and PLGA in particular are highly processable making them very attractive for the fabrication of complex structures and the degradation can be tailored from a few weeks to years. PGA processing is more limited due to the limited number of solvents in which the polymer will go into solution. Because of their easily tailored degradation, they have been studied extensively for drug delivery purposes. However, the PLGA and the related homopolymers are rather brittle, lack functionalities other than end groups for chemical modification, and exhibit bulk rather than surface degradation which can produce a non-uniform release profile which is less than ideal for certain drugs. These limitations have motivated the development and study of a number of complimentary polymers.

1.1.2 PCL and PHA—More Elastomeric Materials

Polycaprolactone (PCL) is a degradable polyester that is in the FDA approved monocrystalline suture material sold by Johnson & Johnson. The glass transition

Fig. 1



temperature or T_g of PCL is molecular weight dependent, as is the T_g for polymers generally, but the T_g of PCL is much lower than PLGA (approximately -60°C for PCL as compared to approximately $40\text{--}60^\circ\text{C}$ for PLGA [7]). This means that at room temperature and body temperature, PCL is above its glass transition temperature and behaves in a more elastic manner than PLGA. It is often used as a scaffolding material for cells associated with flexible tissues because it tends to be more flexible. Copolymers of caprolactone and lactic acid are also commercially available and allow one to have further control over the stiffness and degradation. The greater degree of lactic acid in the backbone tends to increase the stiffness and decrease the degradation rate.

Poly(hydroxyalkanoate) (PHA) is an extremely elastic polyester. It is made by bacteria and can be made in plants including corn. It is used as a storage depot by these organisms. The side chains form entanglements that act similarly to cross links. PCL and PHA have been found to be extremely useful when trying to emulate the properties of highly elastic tissues such as arteries and tendon [8].

The modulus or stiffness of a material can play a critical role in angiogenesis [9, 10], so it is helpful to have a range of materials with different stiffnesses for angiogenic applications.

1.2 Hydrogels

Hydrogels are either physically or chemically crosslinked water-soluble polymers that, through crosslinks, become water-swollen network structure. They are generally highly biocompatible due to the enormous amount of water associated with them. Their mechanical properties parallel the properties of soft tissues, and some have been designed to be injected as a liquid which gels in situ [11]. One of the attractions of using hydrogels over the degradable polyesters or other systems is that one can generally visualize the cells within the gels over time, facilitating live imaging. Almost all of the hydrogels described here have some degree of

Fig. 2

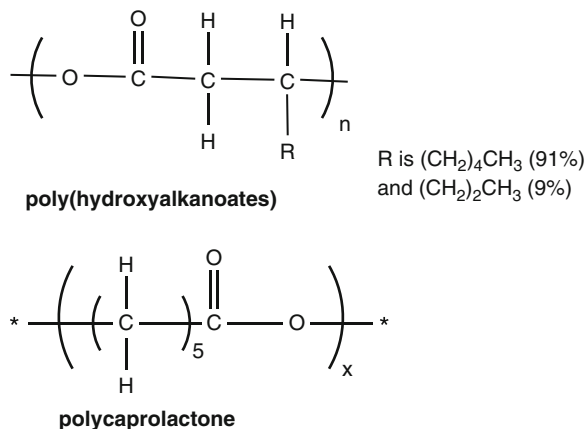
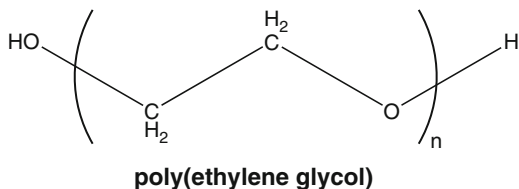


Fig. 3



autofluorescence, but by using cell-labels that are excited at long wavelengths, one can mitigate a great deal of the issue.

One of the best known hydrogels used in three-dimensional cell culture is Matrigel[®]. Matrigel is simple to use, cells can migrate and organize in it [12], and it is commercially available. Another common gel for 3D cell culture is collagen. One can either purchase collagen or do a collagen prep [13]; again, the material is easily obtained and forms a gel readily. The downside of these two gels is that there are strong limits on how much they can be altered. The stiffness can be varied slightly by changing the concentration of the polymer in the gel, but the polymer essentially dictates the chemistry. Synthetic hydrogels or gels based on synthetic and naturally occurring polymers (such as collagen, hyaluronic acid, or other materials) open the door for more variation in the matrix and the potential for directly assessing the role the matrix has on stem cells.

A significant number of synthetic hydrogels used as scaffolds for cells are poly(ethylene glycol) (PEG) -based (Fig. 3). PEG is well tolerated by the body up to a molecular weight of approximately 10,000 g/mole. Above this, the kidneys may have trouble clearing the polymer. It is also soluble both in organics and water making it unique among polymers. In fact, it is highly soluble in water and takes on an extremely hydrated conformation involving an alpha-helical structure with water molecules clustering around the oxygen atoms in the backbone [14]. PEG does not degrade, and hydrogels made from PEG do not degrade unless one uses a degradable linker, peptide, protein, or degradable block such as PLA.

Chemically cross-linked hydrogels may be cross-linked in situ using a chemical initiator [15], or a photoinitiator if the polymer has suitable reactive groups on it such as acrylate groups [2]. The photoinitiated hydrogels use initiators that do not become active until they are exposed to a light of a particular wavelength. The advantage of this over standard initiation schemes is that the hydrogel gelation may be tightly controlled; the un-gelled solution may be injected and then gelled in an controlled manner. These materials also gel quickly, generally in minutes which can be very helpful in encapsulating cells. Photoinitiated hydrogels have been shown to be able to be injected with cells with high cell survival [16, 17] as well as used to deliver growth factors [18].

Physically cross linked hydrogels rely on phase separation of the blocks of block copolymers to gel. The phase separation is generally temperature dependent and reversible. Examples include poly(ethylene)-b-poly(propylene)-b-poly(ethylene) (Pluronics) which has been used in a number of tissue engineering studies [19, 20] and degradable block copolymer systems including ones based on poly(ethylene oxide) and poly(lactic acid) [21, 22].

There are a number of challenges to using hydrogels as the basis for directing stem cells, but the inherent challenges, if addressed, may become the strongest features of using hydrogels to study and control the stem cell microenvironment. Because hydrogels are composed primarily of water soluble polymers, they are very hydrated and typically resist protein adsorption and cell attachment. However, the gels may be chemical modified to include peptides or other moieties to promote cell attachment [16, 23–26]. Therefore, by controlling the concentration of peptides or other groups, one can begin to look at the specific effects of components of the ECM or other molecular structures on stem cell behavior.

1.3 What We Can Model In Vitro with These Systems

Several groups have explored the interactions between endothelial cells and other cell types, the growth factors involved in these interactions, and the role of the matrix molecules in these cocultures [27–30]. These studies have been carried out either in two dimensions in a dish or in vivo or in slice culture. Polymers have the potential to be an excellent intermediate between the reductionist approach of two dimensional culture and the complexities of in vivo or slice systems for investigating interactions [31–34]. Ultimately, these polymer-based approaches may also have therapeutic value in building new vascular systems and several groups look at the constructs with this goal in mind [9, 35–37]. Regardless of one's ultimate goal, being able to select materials with the appropriate properties allows one to move into three dimensions, and there are several systems which are relatively simple that can be employed to take these steps.

Both hydrophobic polymers and hydrogels have been used to grow cells in three dimensions. The former includes polymers such as polystyrene, Dacron, PLA, and PLGA. The latter includes collagen gels, polyacrylamide gels, and PEG gels.

The hydrophobic materials can be processed in a multitude of ways, have a tremendous range of properties, and are capable of being very strong. Hydrogels exhibit properties that are similar to native ECM such as mechanical stiffness but can be more challenging to process and are inherently weaker than many of their hydrophobic counterparts making them challenging at times to handle for cell culture. A few straightforward techniques can help to alleviate some of the challenges.

In tissue culture dishes in two dimensions, endothelial cells are able to organize and form tubes with functional lumen [38]. Endothelial cells are also able to organize and form tubes in collagen. The tube formation assay either in 2D or in collagen in 3D is often used to demonstrate the bioactivity of angiogenic factors [39–42]. Hydrophobic polymers have been used to culture endothelial cells as well and capillary structures have been seen in these materials.

The critical questions become what properties, exactly, do we need and do we want to study with respect to the matrix in three dimensional models. The major properties that have drawn interest with ECM mimics have been the stiffness of the matrix, the presentation (both density and type) of molecules on the matrix, and the pore structure and architecture of the matrix. A number of groups have looked at the role of the elastic modulus on angiogenesis in engineered matrices and found that stiffness does, in fact, impact angiogenesis [9, 10, 43]. There is a balance that needs to be investigated with researcher reporting that very soft matrices with moduli less than 1 kPa may favor angiogenesis when only endothelial cells are cultured [44], but pericytes may require more stiff matrices on the order of 10 kPa or higher [45].

The role of integrins and presentation of peptides for angiogenesis can also be investigated in these model systems under mono- and co-culture conditions [42, 46].

1.4 Moving In Vivo with These Systems: Stable Vessels

A number of groups have started to investigate the role of different matrices and cell types on angiogenesis and vessel stabilization in vivo using the subcutaneous implantation models. The subcutaneous model is extremely easy to perform, allows one to test several implants or formulations per animal, limiting animal numbers, and can be modified to include a coverslip window to facilitate imaging into the implant over time [47].

The caveat to moving in vivo is that one needs to consider the thickness of the implant. The rule of thumb is that an implant needs to be at least 1 mm thick or thicker to be able to investigate the difference between the host response to the implant itself and the impact of the coculture system one is characterizing. It is therefore very important to normalize the thickness of the implants across groups in these studies.

2 Materials

2.1 Commercially Available Polymers

2.1.1 Degradable Polyesters

PGA, PCL, PLGA, and PLA can be obtained from a wide range of producers including Sigma Aldrich, Polysciences, and Boehringer Ingelheim. PHA has become more challenging to obtain, but a relative of PHA, polyhydroxybuterate (PHB) can be obtained from Sigma Aldrich as well as Polysciences.

2.1.2 Water Soluble Polymers

PEG can be obtained from Sigma Aldrich, Polysciences, and a host of chemical companies. Many of these companies also have chemically modified versions of PEG that may circumvent the need for the chemical reactions outlined in the following sections including acrylated versions and versions that are chemically modified to react with free amines. Our lab uses standard PEG and performs the reactions described below because they are simple and reproducible. Some of the activated PEGs that are commercially available seem to be less reactive in our hands than those we synthesize ourselves.

2.1.3 Other Materials

For PEG/PLL hydrogels: Linear poly-ethylene glycol (PEG), N,N'-Carbonyldiimidazole, Dioxane, Argon, Balloon, Syringe, 20 G (1.5 inch) needles, mineral oil bath at 37°C, Spectra-Por dialysis-tubing, 4 l beaker, MilliQ water.

For photopolymerizable PEG/PLL hydrogels: linear PEG 4 K (all OH end groups), methylene chloride (MC) anhydrous, 500 ml round bottom flask, triethylamine (TEA) anhydrous, acryloyl chloride (AC), diethyl ether, dialysis tubing, N,N'-Carbonyldiimidazole (CDI), Dioxane – anhydrous, Argon, Poly-L-lysine MW 1250, Sodium bicarbonate buffer (pH 8.2)

3 Methods

3.1 Fabrication of Hydrogels

3.1.1 Synthesis of PEG/PLL Hydrogels

In our lab, we have developed a PEG/PLL hydrogel. The attraction is that it is a synthetic hydrogel with widely tunable properties that involves extremely simple chemistry. Cells adhere to the continuous network of polylysine. One can also

absorb proteins onto the polylysine network or covalently couple molecules through the free amines on the lysines.

Procedure:

Part I: Making the Positive Pressure Device

1. Select a standard oval balloon and a 5 ml syringe.
2. Remove the stopper from the syringe (put it in the regular trash) and place the end of the balloon over the opening in the stopper end of the remaining syringe.
3. Secure the balloon to the syringe by wrapping Teflon tape around the joint.

Part II: Setup of Water Bath and Thermocouple Hot Plate

1. Fill a glass dish 1/3 with mineral oil.
2. Place dish on hot plate and place thermocouple probe just above the bottom of the dish (do not touch the bottom). Add a stirbar.
3. Turn on the stir and heat switch and set temperature set point on the dial to 150°C
4. When the thermocouple LCD stabilizes, press the + key to 37°C (top right number)
5. Wait for the bath temperature to reach 37°C (center main number)

Part III: Reaction (Perform Only in Chemical Hood)

1. Dissolve 7.5 g PEG in 150 ml dioxane @ 37°C.
2. Determine amount of CDI to add. For every hydroxyl there should be an excess of 8 moles of CDI (FW 162.15).
3. Add CDI to the solution (dissolves quickly) and pour into a 250 ml round bottom flask. Place flask just over stirbar in mineral oil bath.
4. Add a stir bar and cover with a rubber septum.
5. Select 2 **sterile** 20 gauge needles. Use new sterile needles for all applications!
6. Insert one needle of onto the balloon/syringe contraption and into the red septum and using the second needle, fill the balloon/flask with argon until the balloon is about the size of the empty space in the flask.
7. Stir for 2 h at 37°C.

Part IV: Dialysis

1. Dialyze in restriction dialysis tubing (size should allow excess CDI to leave and aPEG to remain) in MilliQ water (watching for abundant bubble formation, and leaving the tops of the tubes open and clipped to the sides of the flask until the bubbles cease to form.)
2. Change the water every 2 h for 6 h and then once a day for 72 h.

3. Freeze down the activated PEG solution in several 50 ml centrifuge tubes (never filling past 35 ml) in liquid nitrogen (covered with kimwipes and rubber bands.)
4. Lyophilize for a couple days.
5. Store dessicated and in the freezer.

3.1.2 Synthesis of Photopolymerized Hydrogels

Part I: Acrylation of PEG (Linear or 4-arm)

1. Dissolve 30 g of PEG in 300 ml of anhydrous MC, pour into bottom of 500 ml round bottom flask with a stirbar
2. Quickly add a 9:1 M excess of TEA to PEG-OH groups
3. Add closed off upper flask and flush both flasks with Argon
4. In the upper flask, add a 1:1 M ratio of AC to PEG-OH groups dissolved 1:10 in anhydrous MC
5. Flush flask with Argon
6. Add solution in upper flask to bottom flask but drop-wise due to the exothermic nature of the reaction
7. Stir for 24 h at room temperature
8. Filter out solid precipitate.
9. Precipitate the PEG in 1.4 l of diethyl ether at 4°C
10. Filter to isolate the precipitate
11. Dissolve the precipitate in deionized water and dialyze in water for 72 h
12. Freeze and lyophilize product

Part II: Activation of ACRL-PEG: Activating PEG with N,N'-Carbonyldiimidazole (CDI)

1. Add 7.5 g acrl- PEG and 150 ml anhydrous-dioxane to a 250 ml round bottom flask.
2. Flush with Ar₂
3. 8:1 M excess of CDI to PEG OH groups (you can actually do ½ since many are acrylated, but I actually just do an larger excess) to solution and flush with Ar₂.
4. Stir for 2 h at 37°C.
5. Dialyze in the restriction dialysis tubing in deionized water for 72 h
6. Freeze and lyophilize
7. Store dessicated at -20°C

Part III: Reacting PLL with Activated ACRL-PEG

1. Dissolve 7 g of PEG with acrylate and activated end-groups in 200 ml
2. 50 mM sodium bicarbonate buffer (pH 8.2)
3. Add drop-wise to 200 mg PLL(1.25 kDa) in sodium bicarbonate buffer.

4. React for 2 h at room temperature stirring constantly.
5. Dialyze in water for 72 h
6. Freeze and lyophilize
7. Store at -20°C

Part IV: Making the Final Gel Product

1. Turn on UV lamp (Black-Ray, UVP B100, 365 nm) – warm up for at least 10 min
2. Prepare a solution of 2.5–5 mg/ml Irgacure 2959 initiator in PBS (protect from light)
3. In a scintillation vial, dissolve polymer initiator solution- vortex until dissolved
4. Place solution under UV light for 4–10 min at 6 mW/cm^2

3.2 *Fabrication of Scaffolds Based on Degradable Esters*

3.2.1 **Fabricating Scaffolds: Salt Leaching**

1. Prepare polymer solution. Typically, we use a 5% w/v solution for salt leaching with the solvent being chloroform (1 g polymer/20 ml solvent = 5% solution).
2. Clean plastic vials. The vials are from Cole Parmer #H-08936-00 (phone 708-647-7600). We usually rinse with CHCl_2 and then H_2O to remove the residual polymer and salt.
3. Weigh 0.4 g of salt/vial. Add and cap vials.
4. Add 0.24 ml solution to each vial. Cap. Tap to level salt and allow to remain capped for 15–30 min to insure salt is level. (You can use the leveling table with level to make sure your scaffolds are flat.)
5. Uncap vials in hood.
6. Allow CHCl_3 to evaporate overnight.
7. Tap out salt-filled scaffolds. They will be very brittle. To get the scaffolds out in one piece, squeeze the containers in one direction, rotate 90° and repeat, then turn over the containers and tap them firmly on the lab bench (on a kimwipe) until they come out.
8. Place the scaffolds in histology cassettes, and place in water (deionized) (2–4 l per 20 scaffolds).
9. Change the water 6X over the course of a day (Approx once per hour).
10. Blot the scaffolds dry.
11. Lyophilize overnight to remove residual water.
12. Store in sealed container with desiccant at -20°C .

3.2.2 **Fabrication of Scaffolds: Planar Freezing**

Dioxane freezes at 11°C . The degradable polyesters are soluble in liquid dioxane but not solid dioxane. By placing the slide on ice one sets up a temperature gradient

between the slide and the surrounding room. The temperature gradient induces the orientation of the dioxane crystals as they form. The solidification expels the polymer. Sublimation of the dioxane using a vacuum system or a lyophilizer leaves behind pores where the dioxane crystallized.

1. Cast 0.4 ml of 5% (w/v) solution of the polymer in dioxane onto a glass slide. I recommend a polymer with a number average molecular weight of 30,000 g/mol or higher. To have the necessary entanglements to stabilize the structure.
2. Place the slide on ice to freeze the dioxane leading to solid-liquid phase separation.
3. After 30–60 s, touch a copper wire which has been immersed in dry ice to the solution to nucleate the freezing process.
4. Once the solution is frozen, place the slides in the freezer at -20 for 1–2 h.
5. Lyophilize the slides. The dioxane is sublimated under vacuum leaving behind a pore structure which is the direct artifact of the solvent crystallization.
6. Remove the scaffolds from the glass. If they are not coming off easily, immerse in water for a few minutes. The slides are very hydrophilic, and the scaffold is very hydrophobic which will help to push the scaffold off of the slide.

3.3 Characterization of Scaffolds

3.3.1 Polymer Characterization

All of the scaffolds here are composed of degradable polymers. While PEG does not degrade, polylysine degrades enzymatically making the gels enzymatically degradable. The degradable polyesters break down by hydrolysis which is acid catalyzed *in vivo*.

The molecular weight of the polymers drops as they are hydrolyzed which impacts the mechanical properties of the materials. Two of the most basic characterization steps for these materials are determining the architecture of the scaffolds since that directly impacts the ability of the cells to migrate through the material and organize, and the mechanical properties since it is becoming increasingly clear that the mechanical properties have a direct impact on cell behavior, and may influence stem cell behavior and differentiation [48].

3.3.2 Scanning Electron Microscopy (SEM)

Scanning electron microscopy is the simplest way to characterize the architecture of a scaffold whether it be a hydrogel based system or a degradable polyester. The basic principle relies on using a filament to create a stream of electrons that one scans across the surface of a material. Electrons are collected to resolve a three

dimensional image. Since polymers are non-conductive, they are usually coated with gold so that the charge does not build up on them.

While there are environmental SEMs in which one can use hydrated materials, the level of hydration is extremely low compared to a hydrogel in normal environments. Therefore, we snap freeze and dry our hydrogels to preserve the architecture of the material.

Preparing a Hydrogel for SEM

Traditional lyophilization dehydrates and collapses the structure of the hydrogel. However, sublimating the water allows the gel to maintain its swollen architecture.

1. Swell hydrogel in deionized water overnight such that gel reaches equilibrium swelling.
2. Freeze swollen gel at -20°C overnight. While it would seem to make sense to freeze the gel faster using liquid nitrogen, we have found that this disrupts the architecture significantly.
3. Place frozen gel in liquid nitrogen. Keep gel in liquid nitrogen until completely cooled, approximately 1 h.
4. Very quickly, place the gel on the lyophilizer on in a vacuum system (pressure of 100 mTorr or less) before melting occurs. Lyophilize until dry.

3.3.3 Mechanical Behavior

Tensile testing is the most common technique. One needs a very small load cell with most of the polymers used in biomaterials. For hydrogels, parallel plate rheology is the most common means to characterize the system. For the degradable polyesters, tensile testing using an Instron system or other tensile system is often the best choice. We find that a load cell of 1 kN is more than adequate for collecting data with most of the scaffolds described here, but a 100 N load cell would also work with some of the more friable systems. The challenge of using a more sensitive load cell is that if one exceeds the maximum load of the load cell, one can damage the system and the costs of such damage are very high.

3.3.4 Instron Testing

The tensile test is the workhorse of mechanical testing. Instron is a company, and their systems are ubiquitous enough, that tensile testing is sometimes referred to as “Instron testing”.

The basic principle is to pull on a sample until it breaks while recording the force and displacement. These can then be converted into stress and strain. The elastic region is the linear, reversible region at low strain, and the plastic region is the irreversible region after the yield stress.

3.3.5 Rheology

Because hydrogels tend to be soft and slippery when hydrated, mechanical testing using an instron can be challenging, even in compression. We have found that the most reliable data can be obtained using a rheometer.

For our hydrogel testing, we use a dynamic frequency sweep test also known as a stress test over a frequency range of 0.01–30 Hz.

3.4 Seeding Cells: General Protocols

The following protocols provide general outlines for approaching cell culture with these materials.

3.4.1 DiI Labeling Cells for In Vitro Experiments

We have found that labeling the cells can allow one to track the cells in the three dimensional scaffolds, particularly the hydrogels.

Preparing DiI Stock Solution

Using CellTracer CM-DiI (C-7001, Molecular Probes) (1 mg solid).

Dissolve 1 mg DiI in 1 ml EtOH.

Aliquot solution in small eppendorf vials (50 μ l/vial).

Store in freezer, away from light.

Making DiI Working Solution

Warm DMEM.

Add 50 μ l DiI stock to 5 ml warmed DMEM.

Filter w/syringe filter (0.2 μ M)

Incubating Cells in DiI Solution

Aspirate medium off 1 T25 dish of cells. Rinse once with PBS.

Add DiI working solution.

Place flask in incubator for 5 min.

Remove flask and place in 4°C fridge for 15 min.

Aspirate DiI solution.

Wash with PBS 2X. Add fresh media. Replace in incubator.

Notes: Protect cells from light at all times. DiI has a relatively short lifetime and high extinction rate. Fixing with formaldehyde should preserve fluorescence of cells.

3.4.2 Coating PEG/PLL Gels with Proteins

Because the gels are charged, proteins adhere to the gels, and one can coat the gels with the protein of interest readily. We have used fibronectin and laminin on the gels with a number of neural cell types.

In the cell culture hood:

1. Prepare PEG/PLL gels with dimensions $1\text{ mm} \times 5\text{ mm} \times 10\text{ mm}$.
2. Sterilize by UV exposure (1 h, 5 mW/cm^2).
3. Prepare protein solution at a concentration of $25\text{ }\mu\text{g/ml}$.
4. Soak gels for 24 h.
5. Wash three times in sterile PBS.

3.4.3 Cell Encapsulation in the Photopolymerizable PEG/PLL Gels

One of the attractions of the photopolymerizable hydrogels is that cells can be encapsulated in the gels.

In the cell culture hood:

1. Sterilize the ACRL-PEG-PLL macromers and glass scintillation under UV light for 30 min.
2. Dissolve Irgacure 2,959 (I2959) (or another cytocompatible photocrosslinking agent) in culture media at a concentration of 5 mg/ml . and sterilized by $0.22\text{ }\mu\text{m}$ filtration. It can be difficult to dissolve. We often make a stock solution of 100 ml by heating the media and I2959 at 37°C for several days.
3. Suspended the cells in a 10% w/v solution of ACRL-PEG-PLL in photoinitiator solution at a final concentration of $5 \times 10^5\text{ cells/ml}$ to 10 million cells/ml.
4. Transfer the cell suspension to a flat bottom glass scintillation vial and place under the UV light (365 nm) for 10 min (6 mW/cm^2).

Remove gels from the scintillation vial and placed into Petri dishes containing 50

3.4.4 Sterilization of Polyester Scaffolds for Seeding

Since PGA, PLA and their copolymers degrade by acid hydrolysis, they will not survive being autoclaved. Commercially available scaffolds arrive unsterilized. While films of these polymers may be successfully sterilized by UV radiation, the UV light will not necessarily penetrate a three dimensional scaffold. Therefore, sterilization by ethylene oxide is the most common technique.

3.4.5 Prewetting of Scaffolds for Seeding

PLA, PLGA, PCL, and PGA are all hydrophobic polymers. Therefore, if one takes a scaffold and immerses it directly in medium, one runs the risk of not having

the medium penetrate the three-dimensional porous network. Therefore, it is recommended that the scaffolds be soaked in ethanol to prewet the scaffolds since ethanol wets the polyesters more than water. Soaking the scaffolds in 70% ethanol overnight has also been used in lieu of the sterilization step with ethylene oxide effectively. Essentially, the scaffolds are placed in 70% ethanol at room temperature overnight. Prior to seeding, the scaffolds are washed three times under sterile conditions in PBS and then are seeded with cells as described in the following sections.

3.4.6 Seeding of Scaffolds: Static Seeding

The easiest method for seeding scaffolds is a static method in which a solution of cells is prepared then dripped onto the scaffold in a small dish. After a few hours of incubation to allow for attachment, more medium is added and the scaffold-cell construct is returned to the incubator.

The following protocol was designed for seeding a porous sponge of PLGA, which was 0.75 mm by 1 mm by 6 mm and was approximately 95% porous.

In a cell culture hood:

1. Transfer the scaffolds from 70% ethanol to a 60 mm dish containing 5 ml sterile PBS.
2. Rinse the scaffolds 3X in PBS. Allow scaffolds to remain in PBS while preparing the cell solution.
3. Trypsinize cells and perform a cell count.
4. Centrifuge and resuspend cells at a concentration of 1 million cells/ml.
5. Place each scaffold in a well of a 12-well untreated cell culture dish.
6. Carefully add in a drop-wise fashion 0.12 ml of the cell solution onto each scaffold.
7. Place scaffolds in incubator for 3 h at 37°C and 5% CO₂.
8. After 3 h, very gently add 1.5 ml fresh medium to each well and carefully replace the scaffolds in the incubator for 24 h
9. After 24 h, aspirate the medium and add fresh medium to each well.
10. Subsequently, change medium approximately every 3 days or as often as needed.

3.4.7 Seeding of Scaffolds: Dynamic Seeding

Dynamic seeding and maintenance affords the opportunity for more uniform coverage and better transport of nutrients and gasses throughout the scaffolds. It is the better choice for the seeding of larger scaffolds. The biggest limitation is that few cells adhere so well to a scaffold that they can be seeded dynamically. One will likely have to modify the scaffolds by coating them with an attachment factor or protein to facilitate dynamic seeding. Even if one cannot seed dynamically, one can often culture dynamically once the cells are attached, and this will permit the greatest transport through the scaffold.

While there are a host of ways to seed and maintain scaffolds in a dynamic environment the following protocol using an orbital shaker in the incubator has been found to be very successful for seeding a variety of scaffolds with neural stem cells and is relatively easy. This protocol uses an excess of cells to obtain well-seeded scaffolds very quickly. One can use a lower cell concentration. As long as the cells attach well to the scaffold, which seems to be the case for a variety of neural stem cells on the PLA and PGA scaffolds, one should get good, uniform cell coverage very easily using this method.

In the cell culture hood:

1. Transfer the scaffolds from 70% ethanol to a dish filled with PBS.
2. Rinse 3X in PBS.
3. Allow the scaffolds to soak in PBS while the cell solution is prepared.
4. Trypsinize the neural stem cells, and resuspend in medium.
5. Centrifuge cells. Perform cell count.
6. Resuspend cells to concentration of 1 million cells/ml.
7. Transfer the scaffolds, one to a well in the 12 well dish.
8. Add 2 ml of the cell solution to each of the wells containing a scaffold.
9. Place scaffolds on orbital shaker set to the lowest speed in the incubator (37°C, 5% CO₂).

One day later, aspirate off the old medium and add 2 ml of fresh medium to each well. Replace the scaffolds in the incubator on the orbital shaker.

10. The medium is then changed every few days or as needed, depending on the size of scaffold and number of cells seeded.

4 Examples of Coculture In Vitro

4.1 Methods

4.1.1 Cells Used

In the coculture experiment described, below, endothelial cells were cultured with neural stem cells. The endothelial cells were isolated from the brain and immortalized [49, 50]. They were maintained in brain endothelial cell medium (DMEM, 10% FBS, 10 mM HEPES, 10⁻⁵ M β – mercaptoethanol, 1% Penicillin/Streptomycin). Green fluorescent protein (GFP) neural stem cells (NSCs) were isolated from whole brains of postnatal day 1 green fluorescent protein (GFP) mice [51–53]. They were maintained in DMEM/F12 media supplemented with 20 ng/ml epidermal growth factor (EGF), 1 mM L-glutamine, 1% N2 supplement, 1% B27, and 1% penicillin/streptomycin with fungizone. EGF is critical for maintaining NSCs in an undifferentiated state [54]. NSCs were passaged (1:2) approximately every 2 weeks [51].

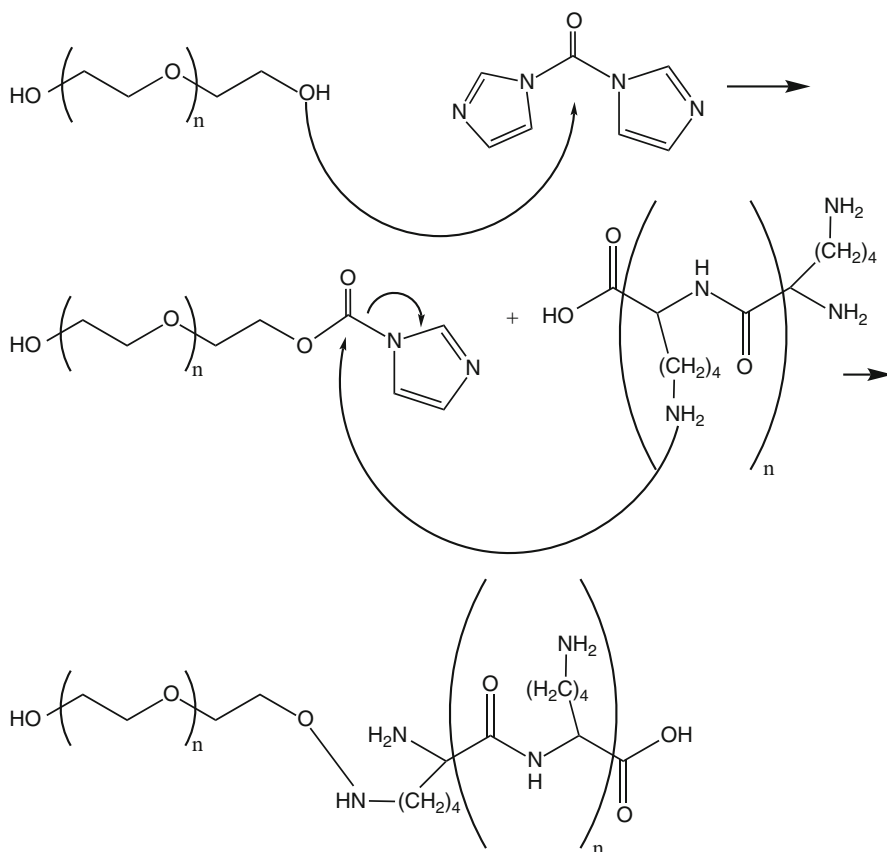


Fig. 4

4.1.2 Hydrogel Preparation

Hydrogel components were sterilized using a 0.22 μm sterile syringe filter prior to crosslinking. The components (activated PEG and polylysine) were mixed in water as described in Sect. 3.1.1 and allowed to gel overnight. Hydrogel disks were cast in scintillation vials and cut using a cork borer to obtain gels that were 1 mm thick and 5 mm in diameter. Gels were sterilized by UV exposure just prior to seeding.

4.1.3 Seeding of Gels

Hydrogel disks 1 mm thick and 5 mm in diameter were seeded with endothelial cells (1 million cells per gel at a concentration of 1×10^6 cells/ml), NSCs (100,000 cells

Fig. 5

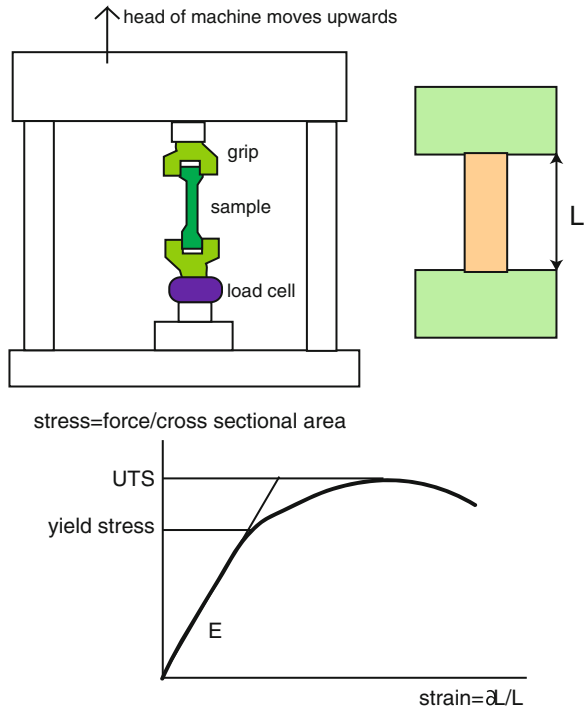
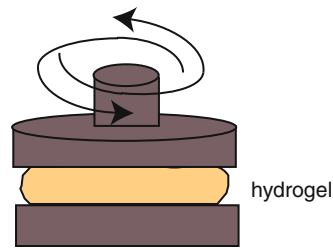


Fig. 6



at a concentration of 1×10^5 cells/ml) , or a co-culture of NSCs:endothelial cells at a ratio of 1:10 (1 million BECs and 100,000 NSCs per implant). Seeded gels were maintained in 12 well plates coated with 1% BSA to reduce cell adhesion to the wells.

For in vitro experiments, seeded gels were maintained under static conditions in the endothelial cell media with a media change every 7 days. Hydrogels were fixed in 10% buffered formalin at 3 days and at 1, 2, 4, and 6 weeks for histological analysis.

4.2 Outcomes

In this set of experiments, one of the most exciting findings was that the cells could organize in the gels. NSCs were found to migrate to the endothelial cells. To further augment the coculture system, we started making the hydrogels with macropores. This involves casting the gels around a salt-leached sponge and then degrading the sponge in 1 N NaOH leaving behind a PEG/polylysine hydrogel with macropores in which the endothelial cells can organize. Details of this and the results can be found in [55].

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