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Use of Hyaluronan-Derived Hydrogels for Three-Dimensional Cell Culture and Tumor Xenografts

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Abstract

The practice of in vitro three-dimensional (3-D) cell culture has lagged behind the realization that classical two-dimensional (2-D) culture on plastic surfaces fails to mirror normal cell biology. Biologically, a complex network of proteins and proteoglycans that constitute the extracellular matrix (ECM) surrounds every cell. To recapitulate the normal cellular behavior, scaffolds (ECM analogs) that reconstitute the essential biological cues are required. This unit describes the 3-D cell culture and tumor engineering applications of Extracel, a novel semisynthetic ECM (sECM), based on cross-linked derivatives of hyaluronan and gelatin. A simplified cell encapsulation and pseudo-3-D culturing (on top of hydrogels) protocol is provided. In addition, the use of this sECM as a vehicle to obtain tumor xenografts with improved take rates and tumor growth is presented. These engineered tumors can be used to evaluate anticancer therapies under physiologically relevant conditions.

Keywords

hyaluronan; semisynthetic extracellular matrix; hydrogel; biodegradable scaffold

INTRODUCTION

This unit describes the use of chemically modified, cross-linkable derivatives of hyaluronan (HA) hydrogels for more physiologically significant in vitro cell culturing and in vivo tumor and tissue engineering applications. Traditional two-dimensional (2-D) culturing conditions lead to aberrant cell behavior that may have limited relevance to in vivo conditions (Roskelley et al., 1994; Weaver et al., 1997; Wang et al., 1998; Cukierman et al., 2001). Mammalian cells do not grow in a physiologically realistic manner on plastic. In vivo, an extracellular matrix (ECM) surrounds the cells in all tissues. The ECM is a complex network of proteins and glycosaminoglycans (GAGS), which form a 3-D microenvironment that plays an integral part in signaling cells to proliferate, migrate, differentiate or invade (Galbraith et al., 1998; Geiger et al., 2001; Lutolf and Hubbell, 2005; Holmbeck and Szabova, 2006).

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Anna Scott is the Director of Operations and equity holder as cofounder for Glycosan BioSystems, Inc.

HA is a major constituent of the ECM and is the only nonsulfated GAG present (Knudson and Knudson, 2001). It is biocompatible and biodegradable, and it performs important biological functions such as stabilizing and organizing the ECM (Fraser et al., 1997; Dowthwaite et al., 1998), regulating cell adhesion and motility (Dowthwaite et al., 1998; Cheung et al., 1999), and mediating cell proliferation and differentiation (Entwistle et al., 1996).

The HA-derived hydrogel (Extracel) discussed in this unit is composed of chemically modified HA containing reactive thiol groups (known as CMHA-S, Glycosil, or Carbylan-S) and chemically modified gelatin containing reactive thiol groups (known as Gtn-DTPH or Gelin-S), which are co-cross-linked with polyethylene glycol diacrylate (PEGDA or Extralink) to form a semisynthetic ECM (sECM; Shu et al., 2004, 2006; Prestwich, 2007, 2008). For clarity and consistency in this unit, we will use the names of the commercially available materials.

All three components of the hydrogel are available as lyophilized solids. Once reconstituted, the solutions can be easily pipetted and transferred into multiple formats (including any well size or tissue culture insert), or it can be injected into an animal model. The hydrogel is formed by mixing the chemical cross-linker, Extralink, with either Glycosil only or Glycosil mixed with Gelin-S. Once the cross-linker is added, the mixture will become more and more viscous until a solid hydrogel is formed. The gelation time can be controlled by the user depending upon requirements.

The HA component Glycosil can be cross-linked alone with Extralink, but most mature cell types do not adhere to HA-only hydrogels. Some cancer cells and stem cells will grow and proliferate in HA-only hydrogels, but usually some attachment factor (e.g., gelatin, an RGD peptide, collagen, laminin, or fibronectin) needs to be mixed with the Glycosil prior to cross-linking with PEGDA. The hydrogel retains proteins greater than 70 kDa in size, so even though the ECM-derived proteins are not covalently attached to the hydrogel, they are entrapped and will only be able to diffuse out as the sECM degrades. Growth factors are also retained within the hydrogel in a similar fashion (Cai et al., 2005; Pike et al., 2006; Riley et al., 2006).

Using three basic components to make an sECM simplifies the biological ECM to a consistent, fully defined, experimentally controllable material for research. Because ECM proteins and growth factors can be incorporated into these hydrogels, it is possible to make a fully defined mimic of specific ECMs found in mammalian tissues if the target tissue ECM composition is known. Additionally, since the basic hydrogel can be formed with only Glycosil and Extralink, animal-free hydrogels can also be made.

STRATEGIC PLANNING

For successful use of these protocols, the researcher must be familiar with how to culture the cells of interest in a 3-D environment or be prepared to conduct several experiments to determine the optimal conditions. Cells cultured in 3-D behave differently than those cultured 2-D on tissue culture-treated plastic. At a minimum, the cell morphology and gene expression patterns can change (Bissell et al., 2003). Because cells receive signals from the matrix on which they are grown (even if this matrix is plastic), the composition and stiffness (compliance) of this matrix help determine the growth and functional characteristics of the cells (Yeung et al., 2005; Engler et al., 2006). In the case of naïve mesenchymal stem cells, the matrix stiffness can cause lineage restriction. For fibroblasts, it changes the amount and arrangement of actin stress fibers (Ghosh et al., 2007). For many cell types, the differences when plating on stiff versus compliant surfaces is not yet characterized. Finally, cells respond differently when encapsulated within a hydrogel or when plated on the surface.

For in vitro cell growth, the culture medium and cell seeding density are very important. It is possible to use the optimal tissue culture–plastic culture conditions as a starting point for the hydrogel experiments. However, it is likely that some modification to these conditions will be required. For the in vivo tumor xenografts, the cell density, injection volume, and hydrogel dilution are critical for the experimental outcome (Liu et al., 2007a). The method of making the hydrogel affects its final properties. There are several variations to this general protocol which are discussed in subsequent sections. Prior to using the hydrogels, the following questions need to be addressed:

1. What gelation time is required?
2. Will cells be encapsulated in the hydrogel?
3. Will ECM proteins be incorporated into the hydrogel?
4. Will growth factors be incorporated into the hydrogel?
5. What hydrogel compliance is required?

Based on the answers to these questions, additional steps may be required in the hydrogel preparation. If it is your first time using these HA-derived hydrogels, performing a simple gelation test before starting the first experiment will greatly improve the chances of success. The test takes ~1 hour and will allow you to understand fundamentally how the materials work. A protocol for performing this test is given in Basic Protocol 1.

This unit contains six protocols which detail how to make the HA-derived hydrogels (Basic Protocol 1), vary their compliance (Basic Protocol 2) and composition (Basic Protocol 3), use them for cell growth in vitro (Basic Protocols 4 and 5), and implant them in mice for in vivo experimentation (Basic Protocol 6).

NOTE: All solutions and equipment coming into contact with cells must be sterile, and proper aseptic technique should be used accordingly.

NOTE: All culture incubations should be performed in a humidified 37°C 5% CO₂ incubator unless otherwise specified.

BASIC PROTOCOL 1: STANDARD HA-DERIVED HYDROGEL PREPARATION

The basic hydrogel, Extracel, is the foundation tool for all the protocols discussed in this unit. Its preparation is required for 3-D cell and pseudo-3-D culture (encapsulation and surface growth) and tumor xenograft experiments. Extracel is composed of Glycosil (thiol-modified HA), Gelin-S (thiol-modified gelatin), Extralink (PEGDA), and degassed, deionized water (DG Water). Glycosil, Gelin-S, and Extralink are available as lyophilized solids. They must be reconstituted using DG Water prior to forming the hydrogel. When reconstituted, they form low-viscosity solutions in phosphate-buffered saline (PBS), pH ~7.4. The hydrogel is formed by mixing all three components together. The gelation time is highly dependent upon the pH of the Extracel solution: the higher the pH, the faster the gelation time. Additionally, depending upon the amount of Extralink used and the concentration of the Glycosil and Gelin-S solutions, gelation will occur in 10 min to >2 hr. Once the Extralink is added there is a time limit on using the hydrogel because it becomes impossible to pipet after the gelation point is reached.

Materials

7.5-ml Extracel Hydrogel Kit (Glycosan BioSystems) containing:

Glycosil (three 1-ml vials)

Gelin-S (three 1-ml vials)

Extralink (three 0.5-ml vials)

DG Water (one 10-ml vial)

Phosphate-buffered saline (PBS; *APPENDIX 2A*)

Serum-free cell culture medium

37°C water bath

1-ml syringes with long-tip 20-G × 1½-in. needles, sterile

37°C shaking or rocking incubator

4-ml glass vials

Prepare the gel

1. Remove Glycosil, Gelin-S and Extralink vials from the −20°C freezer and heat them to 37°C (~30 min).
2. Remove the DG Water from the −20°C freezer and thaw in a 37°C water bath (~15 min).
3. Under aseptic conditions and using a syringe with the exact amount of liquid, add 1.0 ml DG Water to the Glycosil vial. Repeat for the Gelin-S vial.
4. Incubate both vials horizontally at 37°C with shaking (for maximum mixing).
NOTE: Vigorous shaking will speed up dissolving time.
 It will take <30 min for the solids to fully dissolve. Solutions will be clear and slightly viscous.
5. Under aseptic conditions and using a syringe with the exact amount of liquid, add 0.5 ml DG Water to the Extralink vial. Invert several times to dissolve.
6. As soon as possible and within 4 hr of making the solutions, mix equal volumes of Glycosil and Gelin-S in a sterile container. Mix by pipetting up and down gently or inverting the vial.
7. To form the hydrogel, add Extralink to the Glycosil + Gelin-S mix in a 1:4 volume ratio (0.25 ml of Extralink to 1.0 ml Glycosil + Gelin-S).

Perform gelation tests with Extracel

8. Follow steps 1 to 5 (above) for standard hydrogel reagent preparation.
9. Add 0.25 ml Glycosil and 0.25 ml Gelin-S to a small glass vial. Pipet up and down to mix.
10. Add 0.125 ml Extralink to the vial and pipet up and down to mix. Record the time.
 The initial solution of Glycosil + Gelin-S + Extralink will be low viscosity (similar to medium).
11. Every few minutes, invert the vial. Record the time at which the hydrogel no longer flows when the vial is inverted.
 As the hydrogel forms, the liquid will become more viscous.

The gelation time is the difference between the two recorded times. This establishes the maximum length of time you will have to use Extracel after the Extralink is added.

12. Repeat steps 8 to 11, but in addition, add 0.5 ml PBS to the vial of Glycosil + Gelin-S from step 9.

This gelation time will be substantially longer due to the dilution of the Glycosil and Gelin-S.

13. Repeat steps 8 to 11, but in addition, add 50 μ l cell culture medium (no serum or additives) to the vial of Glycosil + Gelin-S in step 9. Pipet up and down to mix.

This gelation time will be about the same as with the first trial (step 11). This simulates the addition of cells in medium into the hydrogel prior to cross-linking.

These gelation tests will help the user determine the time constraints for working with Extracel, once the cross-linker Extralink is added to the Glycosil + Gelin-S. They will also help familiarize the user with how the hydrogel is formed, prior to working with it in an experiment.

BASIC PROTOCOL 2: HA-DERIVED HYDROGEL STIFFNESS VARIATION

As discussed above, hydrogel stiffness can have a dramatic effect on how cells behave in culture. Using the Extracel Hydrogel Kit as per the standard instructions results in a hydrogel compliance of ~100 Pa (J. Vanderhooft, unpub. observ.).

For the HA-derived hydrogels, compliance variation can be achieved in two different ways: (1) varying the concentration of the cross-linker used and (2) varying the concentrations of the Glycosil and Gelin-S solutions.

By increasing the concentration of Extralink, the compliance can be increased to ~500 Pa (Ghosh et al., 2007). Alternatively, diluting the Extracel solutions can decrease it to below the threshold of detection (~20 Pa). Glycosil-only hydrogels cross-linked with Extralink are ~300 Pa. Changing the concentration of Extralink significantly alters the gelation time, as does diluting the Glycosil and Gelin-S solutions. Doubling the Extralink concentration will decrease the gelation time by ~50%. A 2-fold volume dilution will more than double the time for the hydrogel to form.

Materials

7.5-ml Extracel Hydrogel Kit (Glycosan BioSystems) containing:

Glycosil (three 1 -ml vials)

Gelin-S (three 1-ml vials)

Extralink (three 0.5-ml vials; purchase additional vials separately, if required)

DG Water (one 10-ml vial)

Serum-free cell culture medium

Phosphate-buffered saline (PBS; *APPENDIX 2A*), pH ~7.4 and ~7.6

37°C shaking or rocking incubator

37°C water bath

1-ml syringes with long-tip 20-G $\times$ 1½in. needles, sterile

Method 1: vary cross-linker concentration

- 1a. Prepare Glycosil and Gelin-S as in Basic Protocol 1, steps 1 to 4.
- 2a. Under aseptic conditions and using a syringe with the exact amount of liquid, add the appropriate amount of DG Water to the Extralink vial based on the desired hydrogel stiffness (see Table 10.14.1). Invert several times to mix.
- 3a. As soon as possible and within 4 hr of making the solutions, mix equal volumes of Glycosil and Gelin-S by pipetting up and down.
- 4a. To form the hydrogel, add Extralink to the Glycosil + Gelin-S mix in a 1:4 volume ratio (e.g., 0.5 ml Extralink to 2.0 ml Glycosil + Gelin-S).

The gelation time will decrease with higher higher Extralink concentration.

For the stiffest hydrogel, there is insufficient Extralink in the standard Extracel 7.5-ml Hydrogel Kit to use all of the Glycosil and Gelin-S. Individual Extralink vials can be purchased, if required.

Method 2: dilute hydrogel solutions

- 1b. Prepare the hydrogel kit reagents as in Basic Protocol 1, steps 1 to 5 (standard hydrogel reagent preparation).
- 2b. Based on the desired stiffness of hydrogel, aseptically add (using a syringe) varying volumes of sterile PBS to the prepared 1-ml Glycosil and Gelin-S vials (see Table 10.14.2). Invert to mix.
- 3b. Also add varying amounts of sterile PBS to the prepared 0.5-ml Extralink vial (see Table 10.14.2). Invert several times to mix.
- 4b. As soon as possible and within 4 hr of making the solutions, mix equal volumes of Glycosil and Gelin-S by pipetting up and down.
- 5b. To form the hydrogel, add Extralink to the Glycosil + Gelin-S mix in a 1:4 volume ratio (e.g., 0.5 ml Extralink to 2.0 ml Glycosil + Gelin-S).

The gelation time will increase with the solution dilution.

BASIC PROTOCOL 3: ECM COMPONENT INCORPORATION IN HYDROGELS

Gelin-S provides basic cell attachment sites for cell lines and some primary cell types (Shu et al., 2006; Prestwich et al., 2007). However, several cell types are dependent upon specific ECM components for growth and differentiation. For specific cell performance, matricellular and extracellular proteins (e.g., laminin, collagen, fibronectin, vitronectin, aggrecan, decorin) may be added to Glycosil-only hydrogels by the user (Mehra et al., 2006). These proteins are easily incorporated noncovalently into the hydrogel prior to gel formation and retained there after gel formation because of their size. The following protocol describes how to prepare Glycosil-only hydrogels mixed with a laminin isoform from a particular animal source.

Materials

- 1-ml vial of Glycosil (Glycosan BioSystems)
- 0.5-ml vial of Extralink (Glycosan BioSystems)
- DG Water (Glycosan BioSystems)

500 µg/ml commercial (e.g., Sigma) or laboratory-prepared laminin stock solution (or other sterile, cellular matrix protein in aqueous solution): prepared according to the manufacturer's instructions, if commercially obtained

37°C water bath

1-ml syringes with long-tip 20-G × 1½-in. needles, sterile

37°C shaking or rocking incubator

1. Remove the Glycosil and Extralink vials from the −20°C freezer and heat them to 37°C (~30 min). Thaw the laminin solution (for commercial product, per the manufacturer's instructions).

For example, it is necessary to thaw Sigma laminin L6274 overnight.

2. Remove the DG Water from the −20°C freezer and thaw in a 37°C water bath (~15 min).
3. Under aseptic conditions and using a syringe with the exact amount of liquid, add 1.0 ml of DG Water to the Glycosil vial.
4. Place the vial horizontally at 37°C, with shaking (for maximum mixing).

NOTE: Vigorous shaking will speed up dissolving time.

It will take <30 min for the solids to fully dissolve. Solution will be clear and slightly viscous.

5. Under aseptic conditions and using a syringe with the exact amount of liquid, add 0.5 ml of DG Water to the Extralink vial. Invert several times to dissolve.
6. Add 125 µl of commercially obtained or laboratory-prepared laminin to the 1 ml of Glycosil solution. Mix thoroughly.
7. To form the hydrogel, add Extralink to the Glycosil + laminin mix in a 1:4 volume ratio (0.25 ml Extralink to 1.0 ml Glycosil + 0.125 ml laminin).
8. Vary the composition of the hydrogel, as desired, as follows:
 - a. Increase or decrease the amount of laminin.
 - b. Vary the source of the laminin.
 - c. Use other ECM proteins (e.g., a specific type of collagen, fibronectin, vitronectin, decorin) in place of or in conjunction with laminin.

BASIC PROTOCOL 4: CELL GROWTH ON HA-DERIVED HYDROGEL SURFACE

This protocol describes how to make Extracel hydrogels in a 24-well plate format for cell growth on the surface. The protocol can easily be adapted for use with 6-, 12-, 48- and 96-well plates.

Materials

7.5-ml Extracel Hydrogel kit (Glycosan BioSystems)

Phosphate-buffered saline (PBS; *APPENDIX 2A*), sterile

1–2 × 10⁴ cells/ml medium suspension of cultured cells of interest: prepared according to standard procedures (e.g., see *UNIT 1.1*)

Cell culture medium with serum
 0.05% trypsin EDTA (VWR)
 10× collagenase/hyaluronidase (StemCell Technologies)
 37°C water bath
 1 -ml syringes with long-tip 20-G × 1½-in. needles, sterile
 37°C shaking or rocking incubator
 15-ml sterile, conical tubes
 24-well tissue culture plates
 Sterile plate-sealing film (e.g., Axy Seal, VWR) and roller
 Light microscope (10× magnification)

Coat plates

1. Prepare the hydrogel kit reagents as in Basic Protocol 1, steps 1 to 5 (standard hydrogel reagent preparation).
2. Under aseptic conditions and using a syringe with the exact amount of liquid, add an additional 1.0 ml of sterile PBS to both the Glycosil and the Gelin-S vials. Shake to mix.
3. Under aseptic conditions and using a syringe with the exact amount of liquid, add an additional 0.5 ml of sterile PBS to the Extralink vial. Shake to mix.
4. Transfer Glycosil and Gelin-S solutions into a sterile 15-ml conical tube. Mix for at least 3 min with a 25-ml pipet by pipetting up and down.

If the Extracel solutions are not well mixed, the hydrogel surface may not be uniform. This can cause variation in how the cells attach and grow on the hydrogel.
5. Remove a 24-well plate from the packaging.
6. Just before use, add the 1.0 ml Extralink to the tube containing Glycosil + Gelin-S. Mix at least 2 min by pipetting with a 25-ml pipet.
7. Pipet 500 µl into each of ten wells. Rock the plate by hand to ensure that the surface of the plate is equally coated.
8. Remove 300 µl from each well using a pipet, leaving 200 µl of hydrogel in each well. Repeat steps 7 and 8 until all wells are coated.
9. Cover each plate with a sterile film. Seal with a roller so that each well is isolated.

Since these are thin coatings they will dehydrate very easily to form films if they are not completely sealed.
10. Allow gelation to occur on the bench top.

It will take >2 hr for gelation to occur
11. Store up to 4 months at 4°C until ready for use. Do not freeze.

Culture cells on the HA-derived hydrogel surface

12. Remove a precoated 24-well plate from storage at 4°C.

13. Allow it to warm to room temperature or place in the incubator to increase the temperature to 37°C prior to plating.
14. Add 500 µl of the cell suspension in medium to each well on top of the hydrogel.

NOTE: Cells should be cultured in the same medium as when they are grown on tissue culture–treated plastic. This medium may or may not contain serum, depending upon the cell type.

Cell seeding density depends upon the experiment and the cell type. As a rough guideline, follow the cell seeding density used for seeding a tissue culture–treated plastic 24-well plate.
15. Incubate at least 1 hr in a 37°C 5% CO₂ incubator.
16. Verify cell attachment under the microscope. Once confirmed, add the appropriate amount of medium (0.5 to 1.5 ml) to each well and return the plate to the incubator.
17. Change the medium as required (based on changes in the medium's phenol red indicator) by carefully aspirating off the medium.

The hydrogel can easily be removed by vacuum aspiration as well, so this must be done gently and carefully.
18. Pipet 1 to 2 ml medium into each well. Try to avoid disrupting the gel.
19. Return the plate to the incubator.

Recover cells from hydrogel surface

20. Aspirate the medium and wash the hydrogel surface with 1 to 2 ml PBS per well.
21. Add 0.5 ml trypsin solution to the hydrogel surface.

Other products (e.g., Accutase, Detachin, TrypLE) that are gentler than trypsin and are better tolerated by cells can also be used. However they may also degrade the hydrogel so that recovered cells carry some hydrogel particles with them. If this occurs, then use a 10× collagenase/hyaluronidase solution to digest the remaining hydrogel.
22. Incubate at 37°C until the cells begin to detach (~15 min).
23. Gently tap the plate to loosen the cells.
24. Add 2 ml medium with serum to the hydrogel surface and pipet up and down to get a uniform cell suspension.
25. Transfer the cells to a 15-ml culture tube. Add 8 ml medium with serum (10 ml final volume). Centrifuge the cells 5 min at 120 × g, room temperature.
26. Remove the supernatant and replace with 1 to 2 ml fresh medium.

Cell viability will be similar to that of cells grown on plastic and detached with trypsin.

BASIC PROTOCOL 5: CELL ENCAPSULATION IN HA-DERIVED HYDROGELS

Encapsulating cells in HA-derived hydrogels and growing them in tissue culture inserts is the best way (in the absence of a bioreactor) to mimic *in vivo* conditions *in vitro*. This protocol describes how to make Extracel hydrogels in a 24-well plate format, using tissue culture inserts. Other insert formats also work, but the amount of hydrogel used per insert should be varied based on the insert volume.

It is not always necessary to recover cells from the hydrogels. Cells cultured by encapsulating them in tissue culture inserts can be treated like tissue. The hydrogel can be removed from the insert, embedded in paraffin, sectioned, and stained as per standard protocols. Note that small molecule dyes and stains that are less than 70 kDa in size will freely diffuse into the gel.

It is not possible to perform direct antibody staining of cells encapsulated in HA-derived hydrogels because the antibodies are too large to permeate the gel. If embedding, sectioning, and staining is not desirable, then the cells must be recovered from the hydrogel.

Materials

- 7.5-ml Extracel Hydrogel Kit (Glycosan BioSystems) containing:
 - 10 × collagenase/hyaluronidase (Stemcell Technologies)
- Sterile phosphate-buffered saline (PBS)
- ~0.4–2 × 10⁴ cells/ml medium suspension of cultured cells of interest: prepared according to standard procedures (e.g., see *UNIT 1.1*)
- Cell culture medium with and without serum
- 37°C water bath
- 1-ml syringes with long-tip 20-G × 1½-in. needles, sterile
- 37°C shaking or rocking incubator
- 24-well plate with tissue culture inserts (e.g., 6.5-mm Costar tissue culture–treated polycarbonate membrane polystyrene plates, Coming; 8.0-μm pore size Millicel, Millipore)
- 35-mm sterile petri dishes
- Surgical scalpel
- 15-ml conical centrifuge tube

Encapsulate cells

1. Prepare hydrogel reagents as in Basic Protocol 1, steps i to 5 (standard hydrogel reagent preparation).
2. Determine the volume of suspension required to obtain the desired seeding density in 2.5 ml of the hydrogel.
 - Seeding density varies with cell type, but a typical range is 10,000 to 50,000 cells per insert.
3. Prepare two 24-well plates with tissue culture inserts by removing them from their sterile packaging.

4. Mix 1.0 ml of Glycosil and 1.0 ml of Gelin-S.
5. Centrifuge the volume determined in step 2 for 5 min at $120 \times g$, room temperature, and discard the supernatant. Resuspend the cell pellet in the 2 ml of Glycosil + Gelin-S.
6. Just before pouring the hydrogels, add 0.5 ml of Extralink to Glycosil + Gelin-S with cells. Mix completely by pipetting up and down.

Once the Extralink is added you have <20 min before the hydrogel forms.

7. Quickly pipet 100 μ l of Extracel mix into each insert.
Do not add medium at this point because this will dilute the hydrogel and prevent it from gelling.
8. Incubate the plates for ~1 hr in a 37°C, 5% CO₂ incubator to allow the Extracel to gel.
9. Remove the plates from incubator and verify that the hydrogel is solid. If so, add 1.8 ml medium (with serum, if required) to each well. Incubate in a 37°C, 5% CO₂ incubator.
10. Change the medium as required:
 - a. Move each tissue culture insert to an adjacent empty well.
 - b. Aspirate the medium.
 - c. Tap each insert carefully to remove the medium above the hydrogel in the insert.
Aspiration can be used to remove the medium, but, the gel can also easily be removed by vacuum aspiration, so this must be done gently and carefully.
 - d. Replace the insert into its original well.
 - e. Slowly and carefully pipet 1.8 ml medium into each well.
Try to avoid disrupting the gel.
 - f. Return the plate to the 37°C, 5% CO₂ incubator.

Cells behave differently when cultured in 3-D than when grown on the surface of a hydrogel or tissue culture-treated plastic. The cells will grow at different rates (typically slower) and have different morphologies (depending upon the hydrogel stiffness and composition). Additionally, the cells are not passaged in the traditional manner. Since the volume of the hydrogel provides a large volume for growth, the cultures can be maintained for many days, even weeks, before the cells become confluent.

Recover encapsulated cells

11. Dilute the 10 $\times$ collagenase/hyaluronidase 10-fold in the cell culture medium (without serum) used to cultivate the cells.

Do not use undiluted enzyme because this results in low cell viability.

If using medium that contains serum for culture, make sure to wash the hydrogels with serum-free medium or PBS before starting the digestion process because the serum will inactivate the enzymes. At a minimum, wash hydrogels twice for 1 hr to clear serum).

12. Remove the tissue culture insert from the 24-well culture plate. Place upside down in a petri dish.
13. Remove the membrane by using a surgical scalpel to cut it loose from the insert.
The membrane will stay attached to the insert, but usually flips up out of the way.
14. Turn the insert right side up and using the back of a 10- μ l pipet tip punch the hydrogel out of the insert into the petri dish.
15. Place the hydrogel in a 15-ml conical tube and add 5 ml diluted collagenase/hyaluronidase solution to the hydrogel for each 100 μ l of hydrogel.
16. Incubate overnight at 37°C, with gentle shaking.
At the end of the incubation there will still be some hydrogel left in the tube.
If the 10-fold dilution of 10 $\times$ collagenase/hyaluronidase is not satisfactory, try a 5-fold dilution with digestion overnight.
Be cautious about mechanically breaking up the hydrogel prior to digestion because this can lower cell viability significantly.
17. Centrifuge in the conical tube 5 min at 120 $\times$ g, room temperature.
18. Aspirate and discard the supernatant.

Wash the cells

19. Add 5 ml PBS to wash the cell pellet.
20. Repeat steps 17 and 18.
21. Resuspend the cell pellet in 5 ml PBS.
NOTE: *In the PBS you can see any remaining hydrogel.*
22. Centrifuge cell suspension 15 min at 120 $\times$ g, room temperature.
23. Aspirate and discard the supernatant.
24. Add 5 ml medium and repeat the centrifugation.
25. Aspirate off all medium but ~0.5 ml and resuspend pellet (in the remaining 0.5 ml) in the desired volume of cell culture medium.

BASIC PROTOCOL 6: HA-DERIVED HYDROGELS FOR TUMOR XENOGRAPTS

Clinically relevant cancer models are necessary to improve our ability to successfully treat the disease. Anticancer drug discovery efforts require models that can predictably translate preclinical results to efficacy in human patients. Most commonly used are the human tumor xenograft models, where human cancer cells are injected into immune compromised mice. Typically these cells are injected in serum-free medium or buffer or Matrigel (a tumor-

derived basement membrane extract). Poor “take” is often a problem, and many cell lines or patient-derived cells will not form tumors by injection in buffer or medium.

HA-derived hydrogels can be used for the delivery and growth of cancer cells in vivo for the growth of orthotopic and subcutaneous tumors. Using a hydrogel to deliver cancer cells can offer several advantages (Liu et al., 2007a):

The incidence of cancer formation is increased and variability in tumor size is reduced.

The growth of organ-specific cancers is enhanced with improved tumor-tissue integration.

Vascularization is increased and necrosis is reduced in tumors.

Cancer seeding on adjacent tissues or organs is minimized.

The general animal health is improved, leading to better data with fewer animals.

Use the pilot study described below to determine the optimal:

Extracel dilution factor

Cell density

Hydrogel injection volume

Coordination of surgical or injection manipulations with hydrogel handling.

The protocol provided below is based on nine mice with two subcutaneous injections each (see Table 10.14.3), where each experimental condition (cell density and injection volume) has an “ $n = 3$ ” (see Table 10.14.4). Please adjust this protocol (cell density and injection volume, especially) as required, based on experimental requirements and experience.

NOTE: These guidelines describe how to prepare Extracel hydrogels for encapsulation of cancer cells and injection of this suspension into experimental animals *for research purposes only*.

NOTE: Researchers are responsible for obtaining a valid Institutional Animal Care and Use Committee (IACUC) protocol prior to initiation of any experiments (if applicable). The guidelines below only pertain to the operational use of the Extracel product in order to assist in preparing an IACUC protocol.

NOTE: We recommend conducting a benchtop study with Extracel to confirm the Extracel characteristics prior to initiating animal experiments and gain familiarity with handling and timing of use. The gelation time and final hydrogel properties are highly dependent upon the medium used, extent of hydrogel dilution, and final hydrogel pH (see Basic Protocol 1, steps 8 to 13).

NOTE: We *strongly* urge researchers to conduct pilot animal studies to optimize experimental conditions and familiarize the researcher with the handling of Extracel prior to doing large-scale animal testing. The pilot study will provide important information on the time course for tumor growth from a given cell line or primary tumor source, optimal injection size, cell concentration, and Extracel dilution.

Materials

Extracel Hydrogel kit (Glycosan BioSystems) containing:

Glycosil

Gelin-S

Extralink
 DG Water
 Tumor cells
 Cell culture medium (without serum)
 Research animals
 Iodine and 70% (v/v) ethanol and sterile swabs
 37°C water bath
 1-ml syringes with long-tip 20-G × 1½-in. needles, sterile
 37°C shaking or rocking incubator

Prepare hydrogels

1. Remove Glycosil, Gelin-S, and Extralink vials from the −20°C freezer and heat them to 37°C (~30 min).
2. Remove the DG Water from the −20°C freezer and thaw in a 37°C water bath (~15 min).
3. Under aseptic conditions and using a syringe with the exact amount of liquid, add 1.0 ml of DG Water to the Glycosil vial. Repeat for the Gelin-S vial.
4. Place both vials horizontally at 37°C, with shalung (for maximum mixing).
 NOTE: *Vigorous shaking will speed up dissolving time.*
 It will take <30 min for the solids to fully dissolve. Solutions will be clear and slightly viscous.
5. Under aseptic conditions and using a syringe with the exact amount of liquid, add 0.5 ml DG Water to the Extralink vial. Invert several times to dissolve.

Prepare cells

6. Prepare cells for encapsulation by resuspending them in the relevant sterile cell culture medium (without serum) to the appropriate cell density and volume (5×10^7 cells/ml for 100-μl and 200-μl injections and 5×10^6 cells/ml for 100-μl injections).

This protocol assumes that a suspension of 100 μl or 200 μl of Extracel + cells will be injected into nine research animals.

The cell loading and amount of injected Extracel hydrogel used depends upon the application. The amounts discussed in these guidelines are based on published tumor xenograft experiments (Liu et al., 2007a; Prestwich et al., 2007; Prestwich 2008), where a cell concentration of 5×10^7 cells/ml was employed. Lower concentrations may also be effective; however, they will require longer tumor formation times.

Prepare animals and inject cell suspensions

7. Prepare the research animals for surgery as dictated by an approved IACUC protocol and sterilize the sites for surgery with iodine and alcohol swabs.

For subcutaneous injections, the hydrogel with cells is injected under the skin. It is possible to perform two injections per animal, one on each side.

For orthotopic (surgically implanted) injections, the animal is opened surgically and the hydrogel with cells injected into the desired location (e.g., onto the pancreas).

8. Add the appropriate volume of cell suspension to the appropriate amount of Glycosil + Gelin-S (see Tables 10.14.4). Mix the resulting suspension by gently vortexing or pipetting.

The exact time for the hydrogel to become viscous and gel depends on the dilution factor of Extracel and the pH value of the hydrogel solution.

The pH of medium used to dilute the Extracel and the dilution factor can profoundly affect the gelation time. As provided by the manufacturer, the gelation time is ~20 min at ambient temperature. However, the greater the dilution factor, the longer the gelation time.

The pH of Extracel as provided by the manufacturer is controlled to be approximately 7.4 prior to cell encapsulation and further dilution. However, the cell culture medium used can increase or decrease the pH and change the gelation time. For Extracel, a higher pH results in a faster gelation time. For multiple injections, many researchers desire a slower gelation time of 60 or more min. Lowering the pH by 0.1 or 0.2 units, to pH 7.3 or 7.2, combined with dilution with medium, allows researchers to identify an optimal pH/dilution condition for their specific operational needs.

If stiffer hydrogels are required, increase the concentration of Extralink used or decrease the subsequent dilution factor (or resuspend the initial lyophilized Extracel components in half of the indicated water amounts).

Extracel hydrogels form by the reaction of thiols in Glycosil and Gelin-S with the acrylate groups of the cross-linker Extralink. Both Glycosil and Gelin-S can form hydrogels in the absence of Extralink via disulfide bond formation; however, this reaction is normally very slow (hours instead of minutes).

9. When the animals are ready for injection of the hydrogel, add the appropriate amount of Extralink to the cells + Glycosil + Gelin-S. Mix the resulting suspension by gently vortexing or pipetting.

Once the Extralink is added to the Glycosil + Gelin-S + cells, you have between 20 min and 2 hr before the hydrogel forms. Prepare accordingly. If you cannot inject all the animals within this amount of time, consider dispensing aliquots of the cells + Glycosil + Gelin-S into individual injection amounts and adding the Extralink just prior to injection into each animal.

10. Draw the Extracel + cells into a sterile 1-ml syringe with a 20-G needle.
11. Inject the required amount of hydrogel into the research animal at the desired location.

12. After injection, properly care for the research animals and monitor for tumor formation.

COMMENTARY

Background Information

Mammalian tissues are composed of a conglomerate of interconnected cells that perform similar functions within an organism. Cells can interact with each other directly or indirectly, and their activity is modulated by autocrine and paracrine regulatory mechanisms. In epithelial tissues, cells are in close contact with each other. The majority of other tissue types are comprised of cells that are surrounded by a complex network of macromolecules and proteins referred to as the ECM.

Cell culture is a vital tool for basic research in cell biology, drug discovery, drug evaluation processes, and protein biotechnology. Classical tissue culturing techniques were recently proven to be poor mimics of the physiological cellular environment (Bissel et al., 2003, 2005). Currently, two types of 3-D culturing methods are commonly used. One is referred to as 3-D “embedded” cell culture, while the other is known as 3-D “on-top” (Lee et al., 2007). Both methods require an extracellular matrix (ECM) equivalent as the 3-D culturing microenvironment.

At present, the leading ECM equivalent employed for 3-D culture is Matrigel. This is a natural, murine sarcoma–derived product. Its composition includes laminin, collagen, entactin, and growth factors. Matrigel was tested in numerous 3-D cell culturing applications, invasion assays, and tumor xenografts and yielded satisfactory results (Kleinman et al., 1986; *UNIT 12.2*). Nonetheless, Matrigel has drawbacks, the most serious of which pertain to difficulty of use, lack of experimental control of composition, batch-to-batch variability, and lack of utility in translational research for cell therapy (Prestwich, 2007).

A different natural ECM analog, PureCol (consisting of purified type I collagen; Nutracon, <http://www.purecol.nu>), is widely used in cell culture and tissue engineering and as a coating material for medical devices (Elsdale and Bard, 1972; Emerman and Pitelka, 1977; Bell et al., 1979; Schor et al., 1982; Weinberg and Bell, 1986). PureCol has long gelation times (45 to 60 min at 37°C) that make this material unsuitable as a vehicle for 3-D applications. For 3-D encapsulation, the gelation time of PureCol is such that the cells will settle by gravity prior to gelation, so they are not suspended throughout the hydrogel. However, this material is easy to use, has a very long history in cell culture, and is suitable for pseudo-3-D plate coating.

Although naturally derived ECM extracts provide biological recognition and meet key requirements such as presentation of receptor binding ligands and cell-induced proteolytic degradations, they are far from ideal. Issues of limited availability, batch-to-batch variability, pathogen transmission, immunogenicity, technical challenges in handling, and the inability to customize composition and compliance opened the door for a new generation of semisynthetic ECM equivalents.

One such commercially available material is PuraMatrix, a synthetic self-assembling peptide-based material that forms fibrous scaffolds which can be used for 3-D cell embedding or surface plating (Zhang et al., 1995; Holmes et al., 2000; Semino et al., 2003; Bokhari et al., 2005; Yamaoka et al., 2006). This nonanimal-derived material is nonimmunogenic and is suitable for in vivo studies. A major weakness of this material is its preparation protocol; the pH of the initial reagent is 3.0, which strictly limits the time of cell exposure to this environment. Furthermore, the gelation procedure for this material requires

extensive handling. For example, the medium needs to be changed three times in 30 min. Increased handling increases the risk of cell culture contamination and thus limits use to small-scale experimental protocols.

In this unit, we introduced an sECM known commercially as Extracel, a hydrogel based on chemically modified hyaluronan (Glycosil) and gelatin (Gelin-S) that are co-cross-linked with polyethylene glycol diacrylate (Extralink). A generic synthetic scheme for this scaffold is presented in Figure 10.14.1. This biomaterial sustains cell growth and proliferation, while eliminating many of the issues posed by other biomaterials. Its preparation protocol is very user friendly and cell friendly and is suitable for large-scale experimental protocols. The gelation times can be adjusted by varying pH or temperature, and the compliance (stiffness) can be altered by adjusting the degree of cross-linking (Ghosh et al., 2007). In addition, its nature overcomes the issue of immunogenicity in in vivo applications (Liu et al., 2004, 2006a,b, 2007a,b; Duflo et al., 2006; Shu et al., 2006; Orlandi et al., 2007; Prestwich et al., 2007). The biological performance of the four aforementioned ECM equivalents both in pseudo-3-D and 3-D cell cultures were recently compared and contrasted (Serban et al., 2008).

Critical Parameters

The critical parameters required for experimental success were mentioned in each of the protocols. Below, we briefly summarize four key factors that can affect the experimental results:

- Setup time
- Solution pH
- Solution dilution factor
- Cell seeding density.

The last three factors mentioned can be customized to fit experimental requirements.

The duration of material handling is dictated by the chosen properties of the Extracel components (i.e., higher solution pH leads to faster gelation or lower dilution factor causes faster gelation). Although the protocols provided here are intended to serve as a general guide for experimental setup, it is important to recognize that individual cell types and lines might require optimization. For instance, human tracheal scar fibroblasts were found to prefer a gelatin-rich formulation of Extracel (Serban et al., 2008). Based on individual experimental needs, benchtop studies should be conducted to customize the protocols in order to fit the researcher's needs. These trials should only take a short time (a few hours) and can ensure experimental success.

The cell seeding density should be adjusted accordingly, especially when cell will be 3-D encapsulated. It is important to differentiate between surface (2-D) versus embedded (3-D) culturing. To extrapolate an initial 3-D cell seeding density if the 2-D seeding number is known, simply tripling the cell number is a good starting point. Then, work from this cell density to optimize the cell density for a particular experiment. Using classical analytical methods, cell proliferation or viability for both pseudo-3-D or 3-D culturing conditions can easily be determined. Colorimetric (MTS) assays and staining procedures such as fluorescein diacetate propidium iodide (FDA/PI) or hematoxylin and eosin (H&E), are perfectly compatible with Extracel.

Troubleshooting

See Table 10.14.5 for troubleshooting hints for these protocols.

Anticipated Results

For cells grown on the surface of Extracel hydrogels (Basic Protocol 4), you should notice cell attachment in ~2 hr. Cells will elicit a morphology consistent with the hydrogel on which they are grown (Fig. 10.14.2). Cell viability should be similar to the classical 2-D culturing conditions.

Cells that are encapsulated in Extracel hydrogels should be homogeneously distributed in the hydrogel in a 3-D environment (you can monitor this microscopically by changing the focal planes; see Fig. 10.14.3). Cell viability should be similar to the classical 2-D culturing conditions.

For tumor xenografts (Basic Protocol 6), both subcutaneous and orthotopic (surgically implanted) injections should result in well localized, vascularized, and differentiated tumors (Fig. 10.14.4 and Fig. 10.14.5). The use of Extracel as a delivery vehicle for tumor engineering leads to increased incidence of cancer formation, reduced variability in tumor size, enhanced growth of organ-specific cancers, improved vascularization, and lower occurrence of core necrosis and adjacent cancer seeding (Liu et al., 2007a).

Time Considerations

The time considerations for hydrogel handling for each of the six protocols were discussed during the process description. Gelling and incubation times are specific to the applications.

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Figure 10.14.1.

Generic synthetic scheme for Extracel. Extracel is composed of CMHA-S [thiol-modified hyaluronic acid (HA), trade name of Glycosil], Gtn-DTPH (thiol-modified gelatin, trade name of Gelin-S) and PEGDA (polyethylene glycol diacrylate, trade name of Extralink). In this schematic, “linker” refers to the PEGDA molecule. When mixed together, PEGDA chemically cross-links CMHA-S and Gtn-DTPH to form a hydrogel. *NOTE:* each CMHA-S and Gtn-DTPH molecule has multiple modification sites so that the covalent bonds are formed many times on each HA and gelatin molecule. Reprinted from *Methods*, Vol. 45, Serban, M.A. and Prestwich, G.D., Modular extracellular matrices: Solutions for the puzzle, Copyright 2008 with permission from Elsevier.

Figure 10.14.2.

T31 human tracheal scar AM/Ethidium fibroblasts grown on Extracel. Reprinted from *Acta Biomater.*, Vol. 4, Serban, M.A., Liu, Y. and Prestwich, G.D., Effects of extracellular matrix analogues on primary human fibroblast behavior, pp. 67–75, Copyright 2008 with permission from Elsevier.

Figure 10.14.3.

Calcein-homodimer-1 staining of Extracel-embedded T31 fibroblasts (M.A. Serban, Y. Lue, and G.D. Prestwich unpub. observ.) For color version of this figure see <http://www.currentprotocols.com>.

Figure 10.14.4.

Gross view of breast tumors 4 weeks after subcutaneous injection of breast cancer cells in Extracel (reprinted from Liu et al., 2007a).

Figure 10.14.5.

Gross view of colon tumors 4 weeks after subserous injection of colon cancer cells in Extracel (reprinted from Liu et al., 2007a).

Table 10.14.1

Amounts of PBS Added to Extralink When Preparing Hydrogels of Different Stiffnesses by Cross-linker Concentration Variation

Condition	Volume PBS (ml)	Notes
A	0.25	Stiffest
B	0.5	Standard
C	1.0	Softest

Table 10.14.2

Amounts (ml) of PBS Added to Hydrogel Reagent Solutions When Preparing Hydrogels of Different Stiffnesses by Hydrogel Component Dilution

Stiffest		→ Softest			
	Standard	A	B	C	D
Glycosil	0.00	0.25	0.50	0.75	1.00
Gelin-S	0.00	0.25	0.50	0.75	1.00
Extralink	0.00	0.13	0.25	0.38	0.50

Table 10.14.3

Composition of Injections Mixes (µl)

	Glycosil	Gelin-S	Extralink	Cells + medium	Total volume
<i>Six 100- µ l injections</i>					
Injection 1: 90% Extracel + 10% cell suspension	250	250	125	63	688
Injection 2: 50% Extracel + 50% cell suspension	130	130	65	325	650
<i>Three 200- µ l injections</i>					
Injection 1: 90% Extracel + 10% cell suspension	120	120	60	30	330
Injection 2: 50% Extracel + 50% cell suspension	70	70	35	175	350

Table 10.14.4

Pilot Study Conditions

	Cells/ml	Injection volume (μl)
Mice 1–3	5×10^6	100
Mice 4–6	5×10^7	100
Mice 7–9	5×10^7	200

Table 10.14.5**Troubleshooting Guide to Working with Hydrogels**

Problem	Possible cause	Solution
Hydrogel sets too quickly	High solution pH	Adjust solution pH to ~7.4
	Extensive handling time	Dilute solutions
	High solution concentration	Aliquot gel components and cross-link near time of use
Hydrogel sets too slowly	Low solution pH	Adjust solution pH to ~7.4
	Low solution concentration	Reconstitute the lyophilized compounds with less water
Encapsulated cells settle to bottom	High solution dilution	Reconstitute the lyophilized compounds with less water
	Improper cross-linker-to-gel components ratio	Optimize cross-linker-to-gel components ratio
		Add cell suspension to the hydrogel only when mix is becoming viscous
Tumor formation not optimal	Improper solution pH	Adjust solution pH to ~7.4
	Improper solution concentration	Adjust solution concentrations
	Improper cross-linker-to-gel components ratio	Run a pilot, bench-top experiment to determine optimal hydrogel formulation based on experimental needs