Studying Normal Tissue Radiation Effects using Extracellular Matrix Hydrogels

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Abstract

Radiation is a therapy for patients with triple negative breast cancer. The effect of radiation on the extracellular matrix (ECM) of healthy breast tissue and its role in local recurrence at the primary tumor site are unknown. Here we present a method for the decellularization, lyophilization, and fabrication of ECM hydrogels derived from murine mammary fat pads. Results are presented on the effectiveness of the decellularization process, and rheological parameters were assessed. GFP- and luciferase-labeled breast cancer cells encapsulated in the hydrogels demonstrated an increase in proliferation in irradiated hydrogels. Finally, phalloidin conjugate staining was employed to visualize cytoskeleton organization of encapsulated tumor cells. Our goal is to present a method for fabricating hydrogels for in vitro study that mimic the in vivo breast tissue environment and its response to radiation in order to study tumor cell behavior.

Keywords

Cancer Research; Issue 149; Breast cancer; radiation; extracellular matrix; decellularization; hydrogels

Introduction

Cancer is characterized by excess proliferation of cells that can evade apoptosis and also metastasize to distant sites¹. Breast cancer is one of the most common forms among females in the US, with an estimated 266,000 new cases and 40,000 deaths in 2018². A particularly aggressive and difficult to treat subtype is triple negative breast cancer (TNBC), which lacks estrogen receptor (ER), progesterone receptor (PR), and human epidermal growth factor (HER2). Radiation therapy is commonly used in breast cancer to eliminate residual tumor

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Video Link

The video component of this article can be found at <https://www.jove.com/video/59304/>

Disclosures

The authors have nothing to disclose.

cells following lumpectomy, but over 13% of TNBC patients still experience recurrence at the primary tumor site³.

It is known that radiation therapy is effective in mitigating metastasis and recurrence because the combination of lumpectomy and radiation results in the same long-term survival as mastectomy⁴. However, it has recently been shown that radiation treatment is associated with local recurrence to the primary tumor site in immunocompromised settings^{5,6}. Also, it is well known that radiation changes the extracellular matrix (ECM) of normal tissue by inducing fibrosis⁷. Therefore, it is important to understand the role of radiation-induced ECM changes in dictating tumor cell behavior.

Decellularized tissues have been used as in vitro models to study disease^{8,9}. These decellularized tissues preserve ECM composition and recapitulate the complex in vivo ECM. This decellularized tissue ECM can be further processed and digested to form reconstituted ECM hydrogels that can be used to study cell growth and function^{10,11}. For example, injectable hydrogels derived from decellularized human lipoaspirate and from myocardial tissue served as non-invasive methods of tissue engineering, and a hydrogel derived from porcine lung tissue was utilized as an in vitro method of testing mesenchymal stem cell attachment and viability^{12,13,14}. The effect of normal tissue radiation damage on ECM properties, however, has not been investigated.

Hydrogels derived from ECM have the greatest potential for in vitro study of in vivo phenomena. Several other materials have been studied, including collagen, fibrin, and matrigel, but it is difficult to synthetically recapitulate the composition of the ECM¹³. An advantage of using ECM-derived hydrogels is that the ECM contains the necessary proteins and growth factors for a particular tissue^{14,15}. Irradiation of normal tissue during lumpectomy causes significant changes to the ECM, and ECM-derived hydrogels can be used to study this effect in vitro. This method could lead to more complex and more accurate in vitro models of disease.

In this study, we subjected murine mammary fat pads (MFPs) to radiation ex vivo. The MFPs were decellularized and made into pre-gel solution. Hydrogels were formed with embedded 4T1 cells, a murine TNBC cell line. The rheological properties of the hydrogel material were examined, and tumor cell dynamics were evaluated within the hydrogels. Hydrogels fabricated from irradiated MFPs enhanced tumor cell proliferation. Future studies will incorporate other cell types to study cell-cell interactions in the context of cancer recurrence following therapy.

Protocol

Animal studies were performed in accordance with institutional guidelines and protocols approved by the Vanderbilt University Institutional Animal Care and Use Committee.

1. Preparation and ex vivo irradiation of MFPs

1. Sacrifice athymic Nu/Nu mice (8–10 weeks) using CO₂ asphyxiation followed by cervical dislocation.

2. Clean the skin using 70% ethanol.
3. Collect mammary fat pads (MFPs) from sacrificed mice using pre-sterilized scissors and forceps in a 15 mL conical tube containing complete RPMI media (RPMI supplemented with 1% penicillin-streptomycin and 10% fetal bovine serum) (see the Table of Materials).
4. Irradiate samples to 20 Gy using a cesium source.
5. Bring the irradiated MFPs and complete RPMI media into a biosafety cabinet. The media will be dependent on the cell line to be grown in the final hydrogel. Fill 6 cm or 10 cm dishes with enough media to submerge the MFPs. For 6 cm dishes, use 8 mL of media, and for 10 cm dishes, use 20 mL of media.
6. Incubate in a 37 °C/5% CO₂ incubator for two days. The length of time in the incubator may be adjusted.
7. Rinse tissues in phosphate-buffered saline (PBS), blot excess moisture, and place MFPs into 15 mL conical tubes for storage at –80 °C until decellularization. This freezing step aids the decellularization step and the sample should be frozen even if the next steps are otherwise ready.

2. Decellularization (adapted from references^{12,16,17})

NOTE: This procedure was adapted from previously published methods focused on adipose decellularization, which included the sodium deoxycholate ionic detergent rather than sodium dodecyl sulfate to remove DNA efficiently^{12,16,17}.

1. On **day 1**, remove frozen MFPs from –80 °C and thaw at room temperature.
2. Once thawed, dry MFPs briefly on a delicate task wipe. Weigh the MFPs using an analytical scale.
3. Using a pair of forceps with scissors or a scalpel, divide tissue up into 3 mm × 3 mm × 3 mm samples for study of the intact ECM and the remaining tissue for hydrogel production.

NOTE: The number of samples is dependent on the number of testing methods, e.g. the collection of two samples is described below: one for paraffin embedding (step 2.5) and one for freezing in cryostat embedding medium, if desired (see the Table of Materials and step 2.6).

4. Weigh the tissues. If embedding in paraffin for sectioning, continue to step 2.5. If freezing in cryostat embedding medium for sectioning, continue to step 2.6.
5. In a chemical hood, submerge the tissue in 10% neutral buffered formalin (NBF) (see the Table of Materials) for 24 h at 4 °C. Wash 3 times in PBS for 5 min each. Submerge the tissue in 30% sucrose for 48 h at 4 °C.
 1. Weigh the tissue now that this piece has been removed. Continue to step 2.6.

6. In a chemical hood, place MFP pieces in a labeled cassette prepped with cryostat embedding medium. Add more cryostat embedding medium to cover the tissue.
 1. Place the cassette into a beaker of 2-methylbutane (see the Table of Materials) that is pre-cooled with liquid nitrogen. The beaker should have enough 2-methylbutane to cover the bottom but not enough to submerge the cassette because the cryostat embedding medium should not touch the 2-methylbutane. Let the cassette sit in the 2-methylbutane until the cryostat embedding medium freezes and becomes opaque.
 2. Wrap the cassette(s) in foil, label, and leave at -80°C until used for sectioning.

NOTE: Tissues placed immediately in cryostat embedding medium were sectioned at $5\text{ }\mu\text{m}$ while tissues incubated in sucrose were sectioned at $30\text{ }\mu\text{m}$ to retain adipocyte morphology.
7. Use forceps to manually massage the remaining tissue.

NOTE: Tissue pieces may also be placed in 10% NBF for 24–48 h, rinsed in PBS, and left in 70% ethanol until embedding in paraffin. Following embedding, $5\text{ }\mu\text{m}$ sections can be used for hematoxylin and eosin (H & E) staining (see section 7 below).
8. Place the MFPs in 6 cm dishes with 5 mL 0.02% trypsin/0.05% EDTA solution. Incubate at 37°C for 1 h. Spray and wipe the dishes with 70% ethanol before placing in the incubator.
9. Use 0.7 mm strainers to wash the MFPs with deionized (DI) water by pouring water over the tissue three times. Use forceps to manually massage the tissue in between washes.
10. Briefly dry tissue on a delicate task wipe and weigh. Place tissues in a pre-autoclaved beaker containing an appropriately sized stir bar. Cover tissues with 60 mL of 3% *t*-octylphenoxypolyethoxyethanol (see the Table of Materials) per 1 g of tissue and stir for 1 h at room temperature. Use a minimum of 20 mL.
11. Dump tissue and contents into a strainer. Rinse the beaker with DI water and pour onto tissues. Repeat two more times. Use forceps to manually massage the tissue in between rinses.
12. Briefly dry tissue on a delicate task wipe and weigh. Place tissues and stir bars back in the same beakers, and cover with 60 mL of 4% deoxycholic acid per 1 g of tissue. Stir for 1 h at room temperature. Use a minimum of 20 mL.
13. Dump tissue and contents into a mesh strainer. Rinse the beaker with DI water and pour onto tissues. Repeat two more times. Use forceps to manually massage the tissue in between rinses.
14. Briefly dry tissue on a delicate task wipe and weigh.

15. Place tissues in the same beaker with fresh DI water supplemented with 1% penicillin-streptomycin. Cover tightly with paraffin film. Leave overnight at 4 °C.
16. Wash strainers and beakers for use the following day.
17. On **day 2**, drain beaker contents into a strainer. Briefly dry tissue on a delicate task wipe and weigh.
18. Place MFPs in the same beaker with an appropriately sized stir bar. Cover with 60 mL 4% ethanol/0.1% peracetic acid solution per 1 g of tissue. Use a minimum of 20 mL. Stir for 2 h at room temperature.
19. Dump tissue and contents into a 0.7 mm strainer. Use forceps to manually massage the tissue. Place contents back into the beaker. Wash tissue by covering it with 60 mL of 1x PBS per 1 g of tissue. Use a minimum of 20 mL. Stir for 15 min at room temperature. Repeat once.
20. Dump tissue and contents into a 0.7 mm strainer. Use forceps to manually massage the tissue. Place contents back into beaker. Wash tissue by covering it with 60 mL DI water per 1 g of tissue. Use a minimum of 20 mL. Stir for 15 min at room temperature. Repeat once.
21. Briefly dry tissue on a delicate task wipe and weigh. Dump tissue and contents into a strainer. Use forceps to manually massage the tissue.
22. Place contents back into beaker. Cover tissues with 60 mL of 100% n-propanol per 1 g of tissue. Use a minimum of 20 mL. Stir for 1 h at room temperature.
23. Briefly dry tissue on a delicate task wipe and weigh. Dump tissue and contents into a 0.7 mm strainer. Use forceps to manually massage the tissue.
24. Place contents back into beaker. Wash tissue by covering it with 60 mL of DI water per 1 g of tissue. Use a minimum of 20 mL. Stir for 15 min at room temperature. Repeat three times.
25. Dump tissue and contents into a strainer. Repeat steps 2.3–2.6 to collect pieces of tissue for sucrose incubation and freezing in cryostat embedding medium.
26. Briefly dry tissue on a delicate task wipe and weigh. Place in a labeled 15 mL tube. Freeze at –80 °C overnight.

3. Lyophilization

1. Remove the 15 mL tubes from –80 °C and place on dry ice. Keep samples frozen on dry ice until on the lyophilizer.
2. Remove caps. Use a rubber band to attach a delicate task wipe to the top to cover the opening. Put samples on the lyophilizer for at least 2 days.
3. Remove samples from the lyophilizer and place tubes on dry ice. Remove delicate task wipes weigh each sample on an analytical scale. Attach caps and place at –80 °C overnight.

4. Milling

1. Fill a shallow container with liquid nitrogen. Remove samples from the -80°C freezer. Weigh each lyophilized MFP.
2. Place one sample in the mortar. Use a cryogenic glove to hold the mortar in the liquid nitrogen.
3. Use a pestle attached to a handheld drill to mill the sample. Mill in 1 min intervals to check progress and remove gloved hand from liquid nitrogen. Mill for a minimum of 5 min.
4. Repeat for all samples. Spray and wipe the mortar and pestle with ethanol between each sample.
5. Store powdered samples in 15 mL tubes at -80°C until ready for use.

NOTE: Samples may be used immediately or stored overnight.

5. Hydrogel formation

1. If stored at -80°C overnight, remove and thaw at room temperature. While thawing, calculate the necessary weight of pepsin (see Table of Materials) and volume of hydrochloric acid (HCl) needed for each sample that results in a solution with 1% w/v sample powder and 0.1% w/v pepsin in 0.01 M HCl. Add the pepsin to the HCl to form a pepsin-HCl solution.
2. Add sample powder and pepsin-HCl solution to a 15 mL tube. Add a small stir bar, and stir for 48 h.
3. Place the tubes on ice for 5 min. Calculate the necessary volume of 10x PBS needed for each sample that results in a solution with a 1x PBS concentration. Add the appropriate volume of 10x PBS to each tube.
4. Add 10% v/v 0.1 M NaOH to each solution to reach pH 7.4. Use a pH paper to test individually.

NOTE: Gel solution may be stored at 4°C for one week.

6. Encapsulating cells in hydrogels

1. Using GFP- and luciferase-labeled cells
 1. Using the pH 7.4 gel solution, resuspend pelleted GFP- and luciferase-labeled 4T1 cells to a concentration of 500,000 or 1,000,000 cells/mL of gel solution. Add 16 μL of gel-cell solution to each well of a 16-well chamber slide. Incubate for 30 min at 37°C .
 2. Add 100 μL of complete RPMI media to each well. Continue incubation for 48 h at 37°C . The GFP- and luciferase-labeled cells can be visualized using fluorescence microscopy at 0 hours, 24 hours, and 48 hours after gelation.

NOTE: Cell proliferation can be measured for the GFP- and luciferase-labeled 4T1 cells used here by adding (S)-4,5-Dihydro-2-(6-hydroxy-2-benzothiazolyl)-4-thiazolecarboxylic acid potassium salt (see the Table of Materials) to the wells for 10 min and performing bioluminescence imaging using a bioluminescence imaging system (see Table of Materials). Following bioluminescence imaging, the cytoskeleton can be visualized (see section 10).

2. Using unlabeled cells

1. Using the pH 7.4 gel solution, resuspend pelleted unlabeled 4T1 cells to a concentration of 500,000 or 1,000,000 cells/mL of gel solution. Add 16 μ L of gel-cell solution to each well of a 16-well chamber slide and a 96-well plate. Incubate for 30 min at 37 °C.
2. Add 100 μ L of media to each well. Continue incubation for 48 h at 37 °C.

NOTE: Cell viability can be measured (see section 11). The 16-well chamber slide can be used for imaging, and the 96-well plate can be used for quantification.

7. H & E staining

1. For formalin-fixed, paraffin-embedded tissue, submerge slides containing 5 μ m sections twice in 100% xylene (5–10 min) for deparaffinization. Rehydrate by submerging in 100%, 95%, 85%, and 70% ethanol for 5 min each followed by DI water for 5 min. Continue to step 7.6.
2. For frozen tissue sections, take sections immediately from freezer and submerge them in 10% NBF for 10 min. Wash in 1x PBS three times for 5 min each. Rinse in water for 5 min.
3. Stain nuclei with hematoxylin for 3 min. Rinse in running tap water.
4. Differentiate by dipping 1–2 times in 0.3% acid alcohol (0.3% HCl in 70% ethanol). Rinse in running tap water for 5 min.
5. Add bluing agent for 30 s. Rinse in running tap water for 5 min. Submerge in 95% ethanol for 30 s.
6. Incubate with eosin for 90 s at room temperature. Dehydrate in 3 changes of 100% ethanol for 5 min each.
7. Submerge twice in 100% xylene for 5 min each. Add distyrene-plasticiser-xylene (DPX0 mounting media to the slide and add a coverslip. Let it cure overnight before imaging.

8. 1-([4-(Xylylazo)xylyl]azo)-2-naphthol staining

1. Prepare 1-([4-(Xylylazo)xylyl]azo)-2-naphthol solution.

1. Add 0.5 g of 1-([4-(Xylylazo)xylyl]azo)-2-naphthol powder to a beaker with 100 mL propylene glycol. Heat to 95–100 °C while stirring for at least 30 min. Prevent the temperature from exceeding 100 °C.
2. Remove beaker from heat and allow to cool slightly. Pour solution through grade 4 qualitative filter paper to remove any residual particulates.

NOTE: The solution can be stored at room temperature and should be filtered through a 0.45 µm syringe filter immediately before staining.

2. Stain frozen tissue sections.
 1. Remove slides from the –80 °C freezer and air dry for 30 min. Pre-cool 10% NBF at –20 °C for 30 min in a Coplin jar. Fix the slides at room temperature for 10 min. Wash in DI water 3 times for 5 min each.
 2. Remove excess water using a delicate task wipe before immersing in propylene glycol 2 times for 5 min each. Remove slides from propylene glycol. Do not rinse.
 3. Place slides into syringe filtered Oil Red O staining solution for 3 h at room temperature.
 4. Differentiate by placing slides in 85% propylene glycol for 5 min. Rinse samples in PBS twice for 5 min each.
 5. Stain samples with hematoxylin for 30 s. Wash in running tap water for 5 min and then place the slides in DI water.
 6. Mount samples using aqueous based mounting medium and allow media to dry overnight before imaging.

9. Rheology

1. Bring pre-gel solutions from step 5.4 to the rheometer on ice.
2. Attach a 25 mm arm and plate to the rheometer. Open the rheology software on the computer connected to the rheometer.
3. Perform rotational mapping and determine the zero gap. Raise the arm when complete.
4. Pipette 500 µL of pre-gel on the plate. Lower the arm until the pre-gel solution completely occupies the gap between the plate and the arm. Be conservative because lowering the arm past this point will result in pre-gel solution spilling out of the side.
5. Perform a frequency sweep on the pre-gel solution from 0.1–100 Hz after it has remained at 37 °C for 30 min.
6. Raise the rheometer arm when complete. Wipe liquid with a delicate task wipe. Rinse with DI water and wipe with a delicate task wipe. Repeat steps 9.4–9.6 with additional samples.

10. Phalloidin conjugate staining of F-actin

1. Bring phalloidin conjugate solution to room temperature. Briefly centrifuge at a low speed prior to opening. Aliquot enough solution for the assay, and store the rest at -20°C .
2. Dilute 1000x phalloidin conjugate stock solution to a 1x working solution by adding 1 μL stock solution to 1 mL 1x PBS + 1% bovine serum albumin (BSA). This makes enough for 10 wells (100 μL /well).

NOTE: One may use plain 1x PBS, but 1x PBS + BSA is preferred to prevent phalloidin conjugate from sticking to the tube. Do not store this solution after the assay. 1 μL of blue fluorescent dye (see the Table of Materials) working solution may be added to stain for nuclei. Working solution may be made by mixing 1 μL of 20 mM blue fluorescent dye with 11.3 μL 1x PBS.

3. Aspirate media from wells (step 6.1). Rinse wells with 1x PBS.
4. Add 10% NBF. Incubate wells with 10% NBF for 20 min at room temperature. Remove supernatant. Wash 3 times with PBS for 5 min each time.
5. Add 0.1% *t*-Octylphenoxypolyethoxyethanol in PBS for 5 min. Aspirate and wash 3 times with PBS for 5 min each time.
6. Add 100 μL of working solution from step 10.2 to each well. Incubate for 1 h at room temperature. Aspirate and wash 3 times with PBS for 5 min each time. Leave in PBS at 4°C .
7. Aspirate and remove wells from the gasket on the chamber slide. Use needle nose tweezers to remove the gasket from the slide. Mount and coverslip samples using aqueous based mounting medium. Allow to cure (5 min).
8. Observe cells under a fluorescence microscope at excitation/emission of 590/618 nm.

11. Viability assay

1. Rinse the cells with Dulbecco's PBS (DPBS). Add 100 μL of DPBS containing 1 μM calcein AM and 2 μM ethidium homodimer to each well. Incubate for 30 min at room temperature.
2. Image the 16-well chamber slide by fluorescence microscopy. Calcein acetoxymethyl (calcein AM) can be observed at excitation/emission = 494/517 nm. Ethidium homodimer can be observed at excitation/emission = 528/645 nm.
3. Read the 96-well plate on a plate reader using the same wavelengths in step 11.2.

Representative Results

MFPs were decellularized following irradiation using the procedure shown in Figure 1A. MFPs pre-decellularization (Figure 1B) and post-decellularization (Figure 1C) are shown. Decellularization was confirmed using hematoxylin and eosin (H & E) staining, and 1-(4-(Xylylazo)xylyl)azo)-2-naphthol staining was used to evaluate lipid content (Figure 2).

Rheological properties of the ECM hydrogels were also assessed at 37 °C (Figure 3). The storage modulus was higher than the loss modulus for all conditions, demonstrating stable hydrogel formation.

GFP- and luciferase-labeled 4T1 mammary carcinoma cells were encapsulated in the hydrogels. Cell proliferation was examined by fluorescence microscopy, bioluminescence measurements, and viability staining 48 h after encapsulation (Figure 4). Irradiated hydrogels showed an increasing trend in tumor cell proliferation. Phalloidin conjugate was used to visualize F-actin in the encapsulated cells (Figure 5). This technique can be used to examine cell morphology and cytoskeletal properties.

Discussion

This method of hydrogel formation is largely dependent on the amount of starting tissue. Murine MFPs are small, and the decellularization process results in a significant reduction of material (Table 1). The process can be repeated with more MFPs to increase final yield. Milling is another important step that may lead to loss of material. Others have shown success with a cryogenic mill, but this protocol is based on milling via a handheld mortar and electric drill with a pestle attachment^{8,17}. This has the advantage of lower capital costs and minimizing material loss but may introduce variability in the final product.

A challenge to confirmation of decellularization and de-lipidation is in freezing adipose tissue in cryostat embedding medium. Figure 2A shows H & E staining of an unirradiated MFP embedded and sectioned in paraffin. Distinct nuclei are visible on the edges of adipose cells near junctions with other cells, and adipocyte morphology is well-maintained. Figure 2B,C,E,F show MFPs prepared by embedding and freezing MFPs in cryostat embedding medium and sectioning 5 µm slices. This process was unable to retain adipocyte morphology and shape. However, decellularization was confirmed through the loss of nuclei and other traces of DNA (Figure 2C), and de-lipidation was visualized with the loss of neutral lipid content staining (Figure 2F). Adipocyte morphology was maintained by incubating MFPs in sucrose, embedding and freezing in cryostat embedding medium, and sectioning 30 µm slices (Figure 2D,G). While the visualization of H & E staining was difficult with this method (Figure 2D), 1-([4-(Xylylazo)xylyl]azo)-2-naphthol staining confirmed the retention of adipocyte morphology (Figure 2G).

We have developed an in vitro hydrogel model that can mimic the in vivo normal tissue microenvironment and its response to radiation damage. ECM hydrogels have been fabricated in similar studies, but the impact of radiation damage on tumor cell behavior has not been assessed^{9,12,16,17,18}. We expect that irradiation of MFPs will alter ECM remodeling and composition, and these compositional changes will be characterized in future studies. We observed an increasing trend in the proliferation of 4T1 cells within irradiated ECM hydrogels using both bioluminescence imaging and viability staining (Figure 4). In addition, we used phalloidin conjugate to stain F-actin filaments in encapsulated tumor cells and found a qualitative increase in actin alignment and tumor cell elongation in irradiated ECM hydrogels, which suggests an increase in adhesion strength and invasive capacity (Figure

5)^{19,20}. Future experiments will explore changes in focal adhesion dynamics and protease-mediated remodeling for the evaluation of cell migration and invasion.

This method was developed using a murine TNBC cell line, but this method may be used as a platform for evaluating the proliferation and invasiveness of other cell types. Future studies may incorporate immune cells to determine their role in response to radiation as well as other forms of tissue damage (e.g., surgery). Although this study evaluated ECM hydrogels from MFPs irradiated ex vivo, additional studies will explore in vivo radiation of MFPs to evaluate the effect of physiological radiation response and infiltrating immune cells on ECM characteristics. We have established a method to fabricate ECM hydrogels from mouse MFPs to study the effect of normal tissue radiation on tumor cell behavior, and this technique may be extended to human tissue for a more relevant hydrogel model. Overall, examining normal tissue damage through ECM hydrogels may lead to insights into the role of ECM changes following radiation therapy in local recurrence.

Materials

Name	Company	Catalog Number	Comments
10% Neutral Buffered Formalin, Cube with Spigot	VWR	16004-128	-
2-methylbutane	Alfa Aesar	19387	-
AR 2000ex Rheometer	TA Instruments	10D4335	rheometer
Bovine Serum Albumin	Sigma-Aldrich	A1933-25G	-
calcein acetoxymethyl (calcein AM)	Molecular Probes, Inc.	C1430	-
D-Luciferin Firefly, potassium salt	Biosynth Chemistry & Biology	L-8820	(S)-4,5-Dihydro-2-(6-hydroxy-2-benzothiazolyl)-4-thiazolecarboxylic acid potassium salt
DPX Mountant for Histology	Sigma-Aldrich	06522-500ML	-
Dulbecco's phosphate-buffered saline	Gibco	14040133	-
Eosin-Y with Phloxine	Richard-Allan Scientific	71304	eosin
ethidium homodimer	Molecular Probes, Inc.	E1169	ethidium homodimer-1 (EthD-1)
Fetal Bovine Serum	Sigma-Aldrich	F0926-500ML	-
Fisher Healthcare Tissue-Plus O.C.T. Compound	Fisher Scientific	23-730-571	cryostat embedding medium
Fluoromount-G	SouthernBiotech	0100-01	aqueous based mounting medium
FreeZone 4.5	Labconco	7751020	lyophilizer
Hoechst 33342 Solution (20 mM)	Thermo Scientific	62249	blue fluorescent dye
Hydrochloric acid	Sigma-Aldrich	258148-500ML	-
IVIS Lumina III	PerkinElmer	CLS136334	bioluminescence imaging system
Kimtech Science Kimwipes	Kimberly Clark		delicate task wipes

Name	Company	Catalog Number	Comments
n-Propanol (Peroxide-Free/Sequencing), Fisher BioReagents	Fisher Scientific	BP1130-500	-
Oil Red O	Sigma-Aldrich	O0625-25G	1-([4-(Xylylazo)xylyl]azo)-2-naphthol
OPS Diagnostics CryoGrinder	OPS Diagnostics, LLC	CG-08-02	-
PBS (10X), pH 7.4	Quality Biological, Inc.	119-069-151	Phosphate-buffered saline
Penicillin-Streptomycin	Gibco	15140-122	-
Pepsin from porcine gastric mucosa	Sigma-Aldrich	P6887-5G	pepsin
Peracetic acid	Sigma-Aldrich	77240-100ML	-
Phalloidin-iFluor 594 Reagent (ab176757)	abcam	ab176757	phalloidin conjugate
Propylene glycol	Sigma-Aldrich	W294004-1KG-K	-
Richard-Allan Scientific Signature Series Bluing Reagent	Richard-Allan Scientific	7301L	bluing agent
Richard-Allan Scientific Signature Series Hematoxylin 7211	Richard-Allan Scientific	7211	-
RPMI Medium 1640	Gibco	11875-093	-
Sodium deoxycholate, 98%	Frontier Scientific	JK559522	deoxycholic acid
Sucrose	Sigma-Aldrich	S5016	-
Triton x-100	Sigma-Aldrich	X100-100ML	<i>t</i> -Octylphenoxypolyethoxyethanol
Trypsin-EDTA (0.25%), phenol red	Gibco	25200-056	-
Whatman qualitative filter paper, Grade 4	Whatman	1004-110	grade 4 qualitative filter paper
Xylenes (Certified ACS), Fisher Chemical	Fisher Scientific	X5-4	-

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Figure 1: Experimental workflow.

(A) Schematic of hydrogel formation. Digital camera images were taken of MFPs pre- (B) and post-decellularization (C).

Figure 2: Confirmation of decellularization and de-lipidation in MFPs.

Hematoxylin and eosin staining (H & E) of unirradiated MFPs embedded in paraffin and sectioned at 5 μm (**A**) was compared to MFPs frozen in cryostat embedding medium (5 μm sections) before (**B**) and after decellularization (**C**), incubated with sucrose prior to freezing in cryostat embedding medium and sectioned at 30 μm (**D**). 1-([4-(Xylylazo)xylyl]azo)-2-naphthol staining was done to visualize lipid retention in MFPs frozen in cryostat embedding medium (5 μm sections) before (**E**) and after decellularization (**F**) and incubated with sucrose prior to freezing in cryostat embedding medium and sectioned at 30 μm sections (**G**). Scale bars represent 50 μm . Decell = decellularization.

Figure 3: Confirmation of hydrogel formation.

Rheology was used to determine the storage and loss modulus of control (A) and irradiated (B) pre-gel solution made from MFPS at 37 °C and 0.5% strain. Error bars show standard deviation.

Figure 4: Tumor cell proliferation in irradiated ECM hydrogels.

4T1 cell proliferation 48 h after inoculation is shown with pre-gel derived from control (A) and irradiated (B) MFPs. (C) Bioluminescence signal from 4T1 cells embedded within control and irradiated hydrogels. Calcein AM stained live cells and ethidium homodimer stained dead cells were evaluated in control (D) and irradiated (E) hydrogels, and the live/dead ratio was quantified (F). Scale bars represent 200 μm . Error bars show standard error.

Figure 5: Cytoskeletal properties in ECM hydrogels.

Cells within (A) control and (B) irradiated ECM hydrogels are stained with phalloidin conjugate to visualize F-actin (red) and blue fluorescent dye to visualize nuclei (blue) in irradiated MFPs. Scale bar represents 100 μm .

Table 1:
Tissue weights before and after decellularization.

Representative tissue weights for each condition were measured before and after MFP decellularization.

Tissue weight (g)	Control (0 Gy)	Irradiated (20 Gy)
Initial MFP weight	0.461	0.457
MFP weight following histology sample removal	0.423	0.416
MFP weight after decellularization	0.025	0.025
Decellularized MFP weight after histology sample removal	0.015	0.016