Novel decellularization method to generate cell-free extracellular matrices from three-dimensional human gingival fibroblast cultures

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Method Article

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Abstract

Tissue and cell type-specific cell-free extracellular matrices generated by various decellularization protocols can be important tools to study cell interactions with their distinct extracellular niche. Our recent findings have shown that the widely used NH$_4$OH/Triton X-100/DNase decellularization method is not able to completely remove cytoskeletal proteins from long-term three-dimensional human gingival fibroblast cultures. Therefore, we developed a novel decellularization protocol that involves sequential incubation of the cultures with latrunculin B, an actin destabilizing agent, deoxycholate, a mild detergent, and DNase. This protocol efficiently removes cytoskeletal proteins, including actin and beta-tubulin, and DNA from the cultures, which could interfere with the cell-matrix interactions of cells seeded on the decellularized matrices.

Introduction

Decellularization allows for the generation of cell and tissue type-specific three-dimensional (3D) cell-derived matrices (CDMs) synthesized and organized by cultured primary cells. These CDMs can be used to study cell interactions with the extracellular matrix (ECM) by reseeding cells in them. CDMs can also be used to develop novel approaches for regenerative therapy, for instance to promote wound healing$^{1-4}$. Various decellularization methods have been described in literature, and they involve removal of cells and intracellular components using various chemical (such as acids, bases, and detergents), biological (such as enzymes, chelation agents, and toxins), or physical (such as freeze-thaw cycling) agents. In order to avoid protease-mediated degradation during processing, decellularization methods also need to include protease inhibitors to inactivate intracellular proteases released during the cell lysis$^{5,6}$. Decellularization must balance removal of cellular material (such as plasma membrane, actin cytoskeleton, microtubule network, nuclear membrane, and nucleic acids) without significantly compromising the structural integrity and composition of the ECM. However, failing to effectively remove cellular components may attenuate cellular responses and if transplanted in vivo may induce immune responses$^{7,8}$.

The protocols for decellularization are dependent on cell and tissue type and species of origin usually requiring multiple chemical and biological reagents supplied in a series of incubations$^6$. While most of the decellularization protocols were originally developed for tissues, they have also more recently been adapted for CDMs from cultured cells. These CDMs have distinct mechanical properties such as thickness, structure, and density compared to tissue harvests usually requiring optimized reagents, reagent concentrations, and incubation times$^9$.

A widely used, alkaline detergent-based decellularization method has been developed for 3D NIH-3T3 mouse embryonic fibroblast cultures$^{10,11}$. It involves incubation of the cultures in an alkaline detergent
solution (20 mM NH₄OH/0.5% Triton X-100, pH=8.0) to lyse cell membranes followed by digestion with DNase to remove DNA. This decellularization method has been used in a variety of contexts including primary human dermal and lung fibroblast cultures, cancer-associated fibroblast cultures from esophageal, ovarian, renal, pancreatic, prostatic and lung tumors, and murine mammary fibroblasts¹⁰,¹²,¹³. In addition, we have used this method to decellularize 3D cultures of human gingival and skin fibroblasts¹⁴,¹⁵. To this end, primary fibroblasts were seeded at a high density (25,000 cells per cm²) and cultured for 7 days, as originally described for mouse NIH-3T3 cells, to generate 3D cultures¹¹. Analysis of the cultures showed that the cells had formed 3-5 cell layers and were surrounded by their own ECM at day 7¹⁴,¹⁵. However, immunostaining of the decellularized cultures for cytoskeletal actin and β-tubulin showed that the decellularization protocol was unable to remove these cytoskeletal elements completely¹⁵. Therefore, decellularization methods need to be tailored distinctly for different cell types.

Latrunculin B is a toxin isolated from certain sea sponges that binds and destabilizes the actin cytoskeleton by preventing actin filament assembly and actively inducing actin disassembly¹⁶,¹⁷. It has been previously used with DNase and hyper- and hypotonic solutions to decellularize tissue harvests. For example, latrunculin B-mediated decellularization of mouse skeletal muscle results in higher retention of glycosaminoglycan content and improved removal of DNA compared to a trypsin-EDTA method¹⁸. When a latrunculin B-mediated decellularization method, which included a DNase treatment (LAT-D), and a detergent-based (sodium dodecyl sulphate and deoxycholate) decellularization (DET-D) of rat aortic artery and porcine aortic valve was compared, use of LAT-D resulted in similar ECM protein retention and tensile strength, but reduced GAG content compared to DET-D treatment. It was more efficient in eliminating DNA from the cultures while having similar efficacy in removing intracellular proteins including the actin cytoskeleton compared to the DET-D method. Functionally, rat aortic artery CDM yielded via the LAT-D method demonstrated accelerated repopulation following transplantation compared to the DET-D method¹⁶. Therefore, we developed a novel decellularization method of human gingival fibroblast 3D cultures that uses sequential incubations with latrunculin B as an actin destabilizing agent¹⁸, deoxycholate as a mild detergent¹⁹, and DNase to remove cellular elements, including cytoskeletal actin and b-tubulin, and DNA from the cultures¹⁵.

To this end, gingival fibroblasts (seeding density 25,000 cells per cm²) were cultured for 7 days in Dulbecco’s Modified Eagle’s Medium (DMEM) (CAT#31600-083; Gibco Life Technologies, Grand Island, NY, USA), 10% fetal bovine serum (FBS) (CAT#12484-028; Gibco Life Technologies), 1% antibiotic/antimycotic (CAT#15240-062; Gibco Life Technologies), and 50 µg/ml ascorbic acid (CAT#A-4034; Millipore Sigma, Burlington, MA, USA) at 37°C and 5% CO₂ in either 24-well (2 cm²) culture plates on gelatin coated glass coverslips or 6-well (9.6 cm²) culture plates with medium change every two days.
Cultures were then trialed with various concentrations (0.2-5 μM) and incubation times (1-4 hours) of latrunculin B, before subsequent incubations with sodium deoxycholate and DNase (see below). Phase contrast microscopy, staining of DNA by DAPI, and immunostaining of actin and β-tubulin showed that incubation with 0.6 μM latrunculin B for 3 hours\textsuperscript{15} followed by standardized treatment with deoxycholate and DNase\textsuperscript{19} were effective in removing cellular and cytoskeletal elements and DNA from the cultures\textsuperscript{15}. The following decellularization protocol has been tested with gingival fibroblasts cultured up to 14 days as above.

**Reagents**

Latrunculin B (CAT#L-5288; Sigma Aldrich, St. Louis, MO, USA), EDTA-free protease inhibitor (CAT#11873580001; Roche Diagnostics, Indianapolis, IN, USA), phosphate buffered saline (PBS), Tris base (CAT#BP152-1; Fisher Scientific, Waltham, MA, USA), HCl (CAT#258148; Sigma Aldrich), NaCl (CAT#S271-3; Fisher Scientific), MgCl\textsubscript{2} (CAT#M8266; Sigma Aldrich), CaCl\textsubscript{2} (CAT#C-7902; Sigma Aldrich), sodium deoxycholate (CAT#97062-028; Avantor, Radnor, PA, USA), DNase (CAT#3778-0100, Akron Biotech, Sarasota, FL, USA).

**Equipment**

**Procedure**

**Protocol**

1. Prepare stock solutions that will be used in the later steps to prepare sodium deoxycholate and DNase working solutions (see below): 1.0 M Tris-HCl (pH=8), 1.0 M Tris-HCl (pH=7), 1.0 M NaCl, 1.0 M MgCl\textsubscript{2}, 1.0 M CaCl\textsubscript{2}, 5% sodium deoxycholate, and 2 mg/mL of DNase, all in ultra-pure water. The above solutions, except DNase, can be stored at room temperature. DNase stock solution should be stored in appropriate aliquots at -20°C. Note, sodium deoxycholate should be kept in the dark as it is light sensitive. Before use, pass the above stock solutions through a 0.2 μm filter (CAT#76479-058; Avantor).

2. Prepare a working solution of 0.6 μM latrunculin B with 1x EDTA-free protease inhibitor in DMEM. Note, latrunculin B can be stored as a stock solution (1.0 mg/mL in ethanol) at -80°C.

3. Aspirate the medium from cell culture wells and gently wash with PBS.

4. Pipette sufficient volume of the above latrunculin B working solution to cover the cultured cells, for instance 180-200 μL for a 24-well plate well (2 cm\textsuperscript{2}) and 900-950 μL for a 6-well plate well (9.6 cm\textsuperscript{2}), and incubate for 3 hours at 37°C. When observed under a phase-contrast microscope, fibroblasts should appear to be rounding as the actin cytoskeleton is disassembling over the course of the latrunculin B treatment (Figure 1).
5. During the above latrunculin B incubation, prepare the sodium deoxycholate working solution by mixing the above stock solutions in ultra-pure water to achieve final concentration of the solution as follows: 0.05 M Tris-HCl (pH=8), 0.15 M NaCl, 1.0 mM MgCl$_2$, 1.0 mM CaCl$_2$, and 0.5% sodium deoxycholate.

6. Prepare the DNase working solution by mixing the above stock solutions in ultra-pure water to achieve the final concentration as follows: 10 mM Tris-HCl (pH=7), 2.5 mM MgCl$_2$, 2.5 mM CaCl$_2$, and 20 μg/mL DNase. Keep the solution on ice.

7. After the 3-hour incubation, aspirate the latrunculin B solution and wash carefully twice with PBS.

8. Pipette the sodium deoxycholate working solution on the cell layer as above and incubate for 20 minutes at 4°C.

9. After the 20-minute incubation, aspirate the sodium deoxycholate solution and wash once with PBS. Under phase contrast microscope, the cultures should appear to be free of cell bodies displaying only fibrillar ECM (Figure 1).

10. Pipette the DNase working solution and incubate for 20 minutes at 37°C.

11. Aspirate DNase solution and wash twice with PBS. Under phase contrast, no further changes compared to sodium deoxycholate treatment stage should appear (Figure 1).

12. CDMs can be stored in PBS at 4°C for at least one week. To this end, seal the cell culture plates around the sides to reduce evaporation while in storage.

Note: A set of CDMs generated on glass coverslips can be immunostained for actin, beta-tubulin, and DNA to ensure complete removal of these intracellular components.

References


**Figures**

![Image of figures showing untreated culture, LAT B, LAT B + SD, and LAT B + SD + DNase](image)

**Figure 1**

Representative standardized phase contrast microscope images of 14-day 3D gingival fibroblast cultures prior to decellularization (untreated culture) and after sequential incubations with latrunculin B (LAT B; 3 hours), sodium deoxycholate (LAT B + SD; 20 min), and DNase (LAT B + SD + DNase; 20 min). Inserts show higher magnification of select areas.