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Protein-Engineered Hydrogel Encapsulation for 3-D Culture of Murine Cochlea

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Hypothesis: Elastin-like protein (ELP) hydrogel helps maintain the three-dimensional (3-D) cochlear structure in culture.
Background: Whole-organ culture of the cochlea is a useful model system facilitating manipulation and analysis of live sensory cells and surrounding nonsensory cells. The precisely organized 3-D cochlear structure demands a culture method that preserves this delicate architecture; however, current methods have not been optimized to serve such a purpose.
Methods: A protein-engineered ELP hydrogel was used to encapsulate organ of Corti isolated from neonatal mice. Cultured cochleae were immunostained for markers of hair cells and supporting cells. Organ of Corti hair cell and supporting cell density and organ dimensions were compared between the ELP and nonencapsulated systems. These culture systems were then compared with noncultured cochlea.
Results: After 3 days in vitro, vital dye uptake and immunostaining for sensory and nonsensory cells show that encapsulated cochlea contain viable cells with an organized architecture. In comparison with nonencapsulated cultured cochlea, ELP-encapsulated cochleae exhibit higher densities of hair cells and supporting cells and taller and narrower organ of Corti dimensions that more closely resemble those of noncultured cochleae. However, we found compromised cell viability when the culture period extended beyond 3 days.
Conclusion: We conclude that the ELP hydrogel can help preserve the 3-D architecture of neonatal cochlea in short-term culture, which may be applicable to in vitro study of the physiology and pathophysiology of the inner ear. **Key Words:** Hair cells—Organ of Corti—Organotypic—Tissue architecture.
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The organ of Corti is a highly ordered three-dimensional (3-D) structure, which consists of sensory hair cells and surrounding supporting cells. Their precise organization and integrity are required for auditory function. Damage and irreversible loss of hair cells in the mammalian organ of Corti are major causes of hearing loss, a sensory disorder affecting about 48 million Americans (1). There are a variety of diseases and drugs that can impair the organ of Corti, including genetic mutations and ototoxic drugs such as cisplatin and aminoglycosides (2–4).

Whole-organ culture of the organ of Corti provides a widely popular model system to study both the biology and physiology of cochlear cell types and also the pathologic processes affecting them. Specifically, it has been used to examine the effects of growth factors on spiral ganglia neurons (5–8), regulation of cochlear development (9), aminoglycoside ototoxicity (10,11), proliferation of sensory precursors (12), and mechanotransduction (13).

One of the earliest culture systems of the inner ear was the Maximov slide assembly (5,14). Subsequent systems improved on ease of culture and included the collagen floating drop method as well as culture on uncoated and coated surfaces (5,15,16). Many research groups adhere the organ onto a flat surface precoated with an adhesive substrate and submerged under media (16,17). Although this method is simple and provides a viable culture for

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experimentation, it does not adequately preserve the natural complex 3-D structure of the cochlea. For example, affixed organs tend to show distortion over time and become flattened (16,18,19).

Hydrogels have been used in 3-D culture systems for a variety of tissue-engineering applications such as encapsulation of valvular interstitial cells (20,21), embryonic stem cells (22), fibroblast cells (23), endothelial cells (24), and mesenchymal stem cells for osteogenesis and chondrogenesis (21,25,26). An emerging class of hydrogels is protein-engineered scaffolds, which can be designed to mimic many of the properties of native extracellular matrices, including cell adhesivity, mechanical elasticity, and proteolytic degradability (27–29). For example, elastin-like protein (ELP) hydrogels have been successfully used for the encapsulation of dorsal root ganglion, showing the capability to support neurite outgrowth (30), as well as providing a 3-D environment for human embryonic stem cell–derived cardiomyocytes (31). In cochlear cultures, hydrogels have been used primarily as two-dimensional (2-D) surface substrates. 2-Hydroxyethylmethacrylate hydrogels (32), PuraMatrix (33), collagen gels (33), and Matrigel (33) have been used as substrates to promote adhesion of the sensory epithelium to the 2-D surface. As an encapsulation vehicle, Matrigel and collagen gels have been applied only to cultures of the developing otic vesicle (34). These studies support the cytocompatibility of hydrogels with inner ear tissues.

In this study, we generated a novel ELP hydrogel to encapsulate the murine cochlea in vitro. We found that it can preserve the 3-D structure of the tissue while maintaining mechanotransduction properties and cell viability in short-term cultures. Protein-engineered hydrogels therefore may be applied to encapsulate cochlear cultures to preserve tissue architecture.

MATERIALS AND METHODS

ELP Synthesis and Purification

Elastin-like protein was synthesized according to the previously established protocol (35). Briefly, plasmids encoding the ELP sequence were transfected into BL21(DE3) *Escherichia coli*. Elastin-like protein was then expressed under the control of a T7-lac promoter for 3 to 5 hours. Cell pellets were harvested and sonicated. The ELP was subsequently purified by an iterative thermal cycling and centrifugation process to first obtain the ELP in solution (4°C) and then in a pellet (37°C). The purified ELP was ultimately lyophilized and stored as a powder at 20°C.

The hydrogel was designed to allow for tunable cell adhesion and mechanical properties (35). The engineered ELP sequence is composed of an alternating bioactive, cell-adhesive, fibronectin-derived RGD sequence and a structural elastin-like sequence (Fig. 1A). The elastin-like domain contains lysine residues that enable site-specific cross-linking with tetrakis (hydroxymethyl) phosphonium chloride (THPC) to form a hydrogel, with a stoichiometric cross-linking ratio of 1:1 (36). The elastin-like structural domain provides elasticity and resilience to the hydrogel.

Hydrogel Encapsulation of Cochlear Cultures

A schematic of the hydrogel formation as well as encapsulation process is depicted in Figure 1. The lyophilized protein was solubilized overnight in phosphate-buffered saline (PBS) solution (pH 7.4) at 4°C with agitation. The tetrakis (hydroxymethyl) phosphonium chloride cross-linker was dissolved in PBS such that mixing the two components resulted in the formation of a chemically cross-linked hydrogel of 3% (weight/volume) ELP and a 1:1 ratio between active cross-linking sites on the polypeptide chain and the cross-linker molecule. Both ELP and THPC

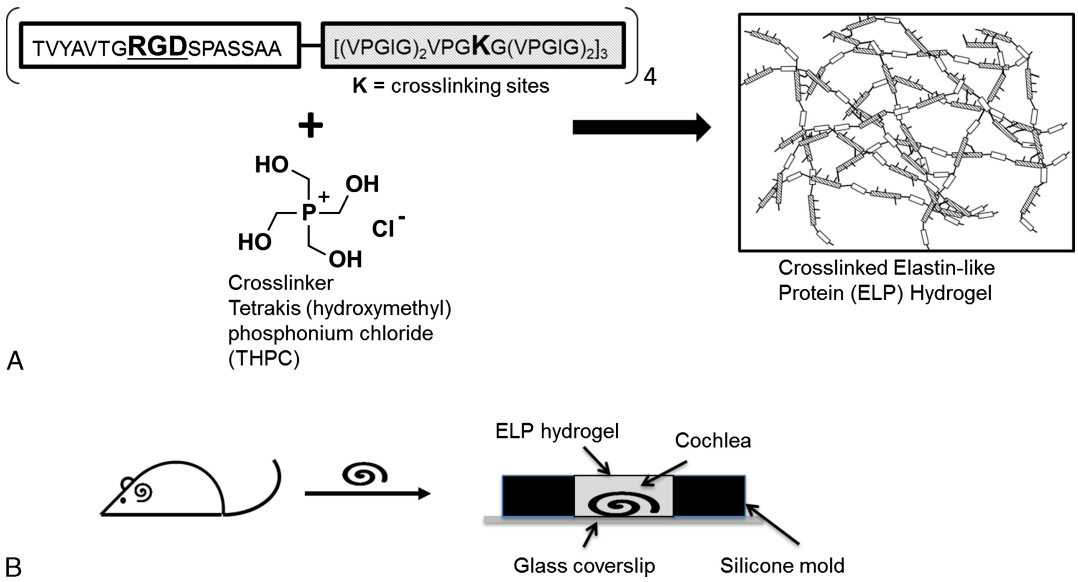

FIG. 1. Elastin-like protein hydrogel design and encapsulation. *(A)* Elastin-like protein is composed of a bioactive cell-adhesive RGD sequence and an elastin-like sequence with cross-linking sites and becomes a cross-linked hydrogel in the presence of the cross-linker THPC. *(B)* Cochleae were dissected from P2-3 wild-type mice, placed within silicone molds, and encapsulated within the 3-D ELP hydrogel.

solutions were sterilely filtered, stored on ice, and mixed immediately before use.

Cochleae were isolated from postnatal 2- to 3-day-old (P2–3) CD1 mice (Charles River, Wilmington, MA, USA) and collected in ice-chilled sterile Hanks' balanced salt solution (Cellgro, Manassas, VA, USA). Two-millimeter inner diameter silicone molds (Electron Microscopy Sciences, Hatfield, PA, USA) adhered onto 10-mm glass coverslips (Fisher Scientific, Hampton, NH, USA) were autoclave sterilized and then precoated with CellTak (BD Biosciences, Franklin Lakes, NJ, USA) before dissection. Isolated cochleae were placed in the 2-mm inner diameter silicone molds and adhered onto the 10-mm glass coverslips. Elastin-like protein and THPC solutions were then mixed. A total hydrogel volume of 2.5 μL was used to encapsulate the organ within the confines of the silicone mold. The 3% ELP hydrogels were polymerized for 10 minutes at room temperature, followed by 10 minutes at 37°C, and then submerged and cultured in DMEM/F12 (Gibco, Carlsbad, CA, USA) supplemented with N2 (1:100; Life Technologies, Carlsbad, CA, USA), B27 (1:50, Life Technologies), ampicillin (50 μg/mL; Sigma, St. Louis, MO, USA), and 10% (vol/vol) fetal bovine serum.

Nonencapsulated cochleae were cultured in the same media with serum as previously described. Isolated cochleae were placed onto 10-mm glass coverslips precoated with CellTak and cultured in media with identical supplements. Cochleae were cultured at 37°C and 5% CO_2, with media changed every 1 to 2 days for both groups.

Live Cell Staining

Viability of cultured cochlea was studied using a commercial Live/Dead assay (Life Technologies). Live cells are labeled with Calcein-AM through intracellular esterase activity while ethidium homodimer-1 labels nonviable cells because of loss of plasma membrane integrity. Cultured cochleae were briefly rinsed with PBS and then incubated with 4 μM calcein-AM and 4 μM ethidium homodimer-1 for 0.5 hours (nonencapsulated) and 1.25 hours (ELP encapsulated) at 37°C, 5% CO_2. Tissues were then rinsed twice with PBS and immediately imaged as a z-stack using confocal microscopy.

The patency of mechanotransducer (MET) channels was studied with FM1-43 dye (Life Technologies) (37). Both nonencapsulated and ELP-encapsulated cultured cochleae were rinsed with prewarmed PBS once and then submerged with 5 μM FM1-43 for at least 15 seconds before imaging. After FM1-43 dye was placed into the culture, live imaging of apex, middle, and base was performed using an inverted confocal microscope (Zeiss LSM 5 Exciter, Germany).

Immunocytochemistry

Encapsulated cochleae were fixed with 4% paraformaldehyde (Electron Microscopy Sciences) in PBS at 37°C overnight. The next day, tissues were rinsed with PBS and then permeabilized with 0.1% Triton X-100 solution in PBS for 30 minutes, and immersion in blocking solution (5% goat or donkey serum, 0.1% Triton X-100, 1% bovine serum albumin, and 0.02% sodium azide [NaN_3] in PBS) at room temperature for 1.5 hours. Samples were then incubated at room temperature overnight with primary antibodies that were diluted in blocking solution. The following day, tissues were rinsed with PBS and then incubated at room temperature overnight with secondary antibodies diluted in 0.1% Triton X-100, 0.1% bovine serum albumin, and 0.02% NaN_3 in PBS. The staining duration was increased for the hydrogel-encapsulated organs to optimize staining quality.

Immunostaining of nonencapsulated cochlea was carried out as previously described (38). Nonencapsulated tissues were fixed with 4% paraformaldehyde in PBS at room temperature for 1 hour (39). Then tissues were immersed in blocking solution at room temperature for 1 hour as before. The cochleae were then incubated with primary antibody in blocking solution at 4°C overnight. The samples were then rinsed with PBS and incubated with secondary antibody as above. Primary antibodies used in the studies include anti-MyosinVIIa (1:1000; Proteus Bioscience, Ramona, CA, USA) and anti-Sox2 (1:400; Santa Cruz, Dallas, TX, USA). Secondary antibodies included Alexa Fluor–conjugated antibodies (1:500; Life Technologies). 4′,6-Diamidino-2-phenylindole (DAPI) (1:1000; Life Technologies) was used for labeling nuclei and Alexa Fluor–conjugated phalloidin was used to stain F-actin (1:1000; Sigma).

The specificity of anti-MyosinVIIa antibody for hair cells was confirmed by phalloidin labeling of hair bundles. Anti-Sox2 antibody specifically labeled supporting cells underneath Myosin7a-expressing hair cells. Anti-MyosinVIIa and anti-Sox2 primary antibodies were omitted in the immunostaining process as negative controls and did not label hair cells and supporting cells, respectively.

Cryosectioning

Heads from P2-P3 mice were isolated and fixed with 4% paraformaldehyde in PBS at 4°C overnight. Tissues were then cryoprotected by successive incubation in 10%, 20%, and 30% sucrose in PBS and then embedded in OCT compound (Sakura Finetek, Torrance, CA, USA). Frozen sections were then performed at 10- to 12-μm thickness with a Sakura cryostat (Sakura Finetek).

Image Analysis

Confocal images of the cochlea were taken using Zeiss LSM 5 Exciter and LSM 5 Pascal microscopes. The apex, middle, and basal regions were separately analyzed. Z-stack images were taken at 1 to 2 μm intervals to span the cochlea. These confocal images were then used to measure the width and height of the organ of Corti. The height was determined by measuring the distance from below the supporting cell layer to just above the hair cell stereocilia. This measurement was made at the inner hair cell and the third outer hair cell positions (Fig. 2). The width was the largest distance spanning the modiolar surface of inner hair cell and the strial surface of the third outer hair cell. Counts of inner hair cells and outer hair cells were performed in images (150 × 150 μm) from individual cochlear turns. Three-dimensional reconstruction of z-stack images was performed using Volocity imaging software (Improvision, Waltham, MA, USA).

Statistics

Two-tailed unpaired Student's *t*-test was performed using Microsoft Excel (Microsoft, Redmond, WA, USA). Values of $p < 0.05$ were considered statistically significant.

RESULTS

Hair Cell Integrity and Mechanotransduction

Cochleae from 3-day-old mice were encapsulated in ELP hydrogel and cultured in serum-enriched media. We administered the styryl dye FM1-43, which has been shown to penetrate the MET channels in hair cells (37). In nonencapsulated cochlea, FM1-43 dye robustly labeled outer and inner hair cells. This is demonstrated in Figure 3A

for nonencapsulated cochlea after 3 days of culture. Hair cells from ELP-encapsulated cochlea were similarly labeled as demonstrated in Figure 3B after overnight culture. Hair cells within the apex, middle, and base of the cochlea were similarly labeled. These results suggest that MET channels remain patent after hydrogel encapsulation.

Immunostaining of nonencapsulated cochlea (Fig. 2A) and ELP-encapsulated cochlea (Fig. 2B) showed no loss of MyosinVIIa-positive hair cells in either system after 3 days of culture.

Structure of the Organ of Corti

Immunostaining of the encapsulated cultured cochlea revealed MyosinVIIa-expressing hair cells crowned with phalloidin-labeled stereocilia on their apical surfaces and adjacent Sox2-positive supporting cells in the organ of Corti. Figure 4 provides a 3-D reconstruction of immunostained organ of Corti after 3 days of culture in nonencapsulated and encapsulated form. Without encapsulation, the organ of Corti appeared flattened and widened after 3 days in culture, and organization of hair cells and supporting cells was distorted (Fig. 4A). Three-dimensional reconstruction showed that hair cells appeared more slanted toward a horizontal position (Fig. 4A), contrasting the more upright orientation in situ (Fig. 4C). Hair cells normally reside in the luminal layer of the organ of Corti, whereas supporting cells are found in the basal layer in contact with the basilar membrane. Nonencapsulated cultured cochleae frequently contained blurring of this layered organization, with supporting cells appearing close to the level of hair cells (Fig. 4A). In contrast, the ELP-encapsulated organ of Corti maintained its normal cellular organization (Fig. 4B), with hair cell orientation and location more closely resembling that of the noncultured organ (Fig. 4C).

To quantify the extent of structural changes in cultured organs, we measured the height and width of the organ of Corti in each cochlear turn. At both the inner hair cell and third outer hair cell positions, the nonencapsulated cultured organ of Corti (3 days in vitro) showed decreased height in comparison with the encapsulated cultured organ. As shown in Figure 5 for the medial and lateral organ of Corti, the height of the cultured encapsulated organ of Corti in the basal turn approximately doubled the height of the cultured nonencapsulated organ of Corti.

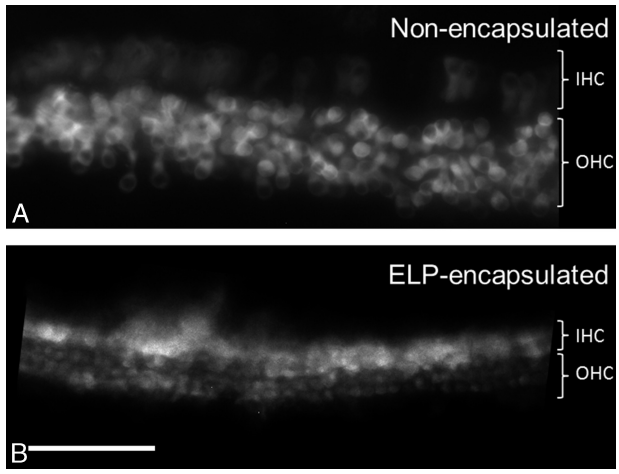

FIG. 3. FM1-43 staining of hair cells in (*A*) nonencapsulated organ of Corti and (*B*) ELP-encapsulated organ of Corti. Scale bar = 50μm.

These differences were similar for all three cochlear turns (apical and middle turns not shown). After 3 days of culture, the nonencapsulated organ of Corti was significantly wider than the ELP-encapsulated tissues ($p < 0.01$). This difference was observed in all three cochlear turns (apical and middle turns not shown).

Control cochlea for structural comparisons included cross-sections from P2-3 noncultured cochlea, in addition to P6 noncultured cochlea fixed and stained in the same manner as the cultured cochlea. Representative comparisons are shown in Figure 5 for the basal turn. The ELP-encapsulated organ of Corti showed a comparable height relative to cross-sections of noncultured cochlear tissues, but more than double that of both the nonencapsulated cultured ($p < 0.01$) and the noncultured tissues ($p < 0.01$). These differences were observed in all three cochlear turns (apical and middle turns not shown). This suggested that ELP encapsulation maintained the height of the organ of Corti during culture, but that the process of mounting the cochlea can flatten the organ. The ELP-encapsulated organ of Corti width was similar to that of the noncultured organ of Corti. However, the nonencapsulated organ of Corti width was significantly larger compared with ELP-encapsulated

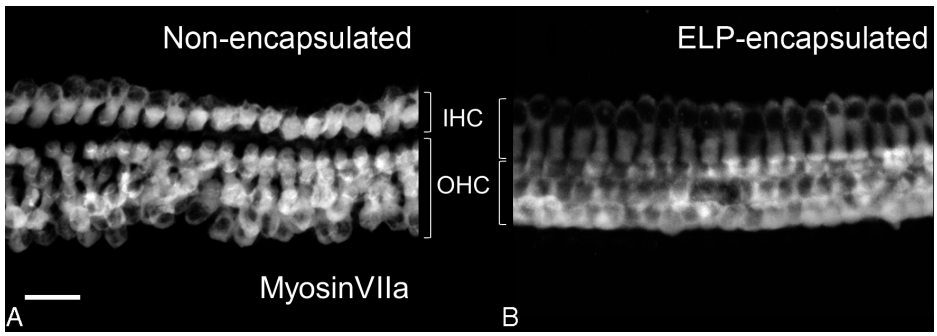

FIG. 2. Projection of z-stack images of whole-mount organ of Corti showing MyosinVIIa-positive inner hair cells (IHC) and outer hair cells (OHC) for (*A*) nonencapsulated and (*B*) ELP-encapsulated cochlea. Scale bar = 20 μm.

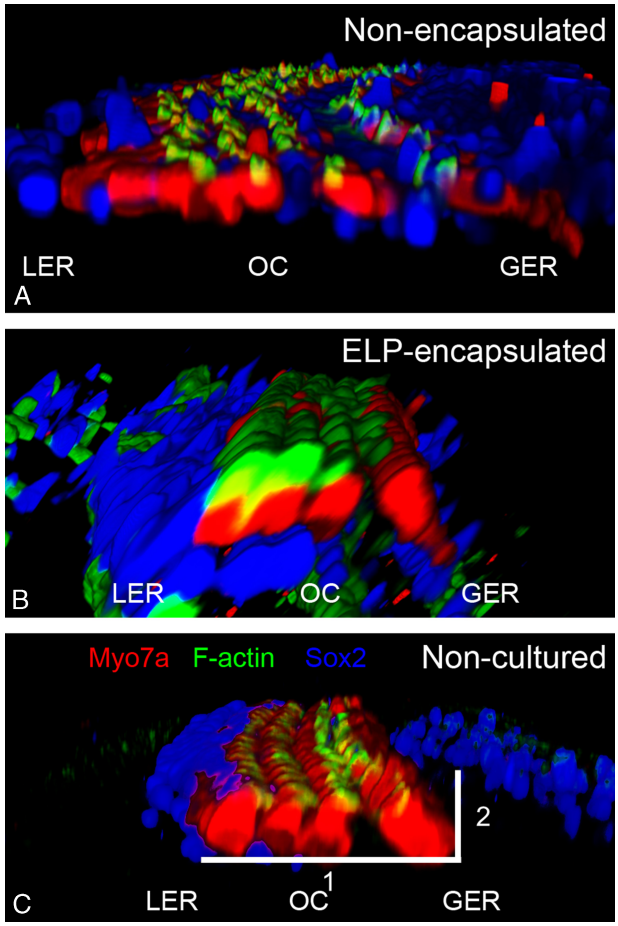

FIG. 4. Three-dimensional representation of *(A)* nonencapsulated cultured organ of Corti, *(B)* ELP-encapsulated organ of Corti, and *(C)* noncultured organ of Corti with immunostaining for F-actin (green), MyosinVIIa (Myo7a, red), and Sox2 (blue). *(C)* Depiction of measurements of width (1) and height of organ of Corti (2). LER indicates lesser epithelial ridge; OC, organ of Corti; GER, greater epithelial ridge.

and noncultured organs ($p < 0.01$). Like the organ of Corti height, the organ width was maintained in the ELP-encapsulated culture system.

The densities of inner hair cells, outer hair cells, and total hair cells in the ELP-encapsulated organ of Corti are comparable to those in the noncultured organ but are significantly greater than in the nonencapsulated organ in the middle and basal turns of the cochlea ($p < 0.05$). This is shown for the basal turn in Figure 6, with similar results observed in all three turns (apical and middle turns not shown). The decrease in hair cell density in nonencapsulated organ of Corti corresponded to the widening and spreading of the organ of Corti during culture without missing hair cells detected. The organ of Corti measurement and hair cell density comparisons suggested that, during 3 days of culture, encapsulation prevented the structural distortion that can occur in nonencapsulated culture.

Viability

Figure 7 shows live (green) and dead (red) staining for a representative complete nonencapsulated and ELP-encapsulated cochlea cultured for 3 days. We did not detect any dead cells within the nonencapsulated organ of Corti cultured for 3 days (Fig. 7A), 4 days, and 6 days (not shown). Cells within the ELP-encapsulated cochlea

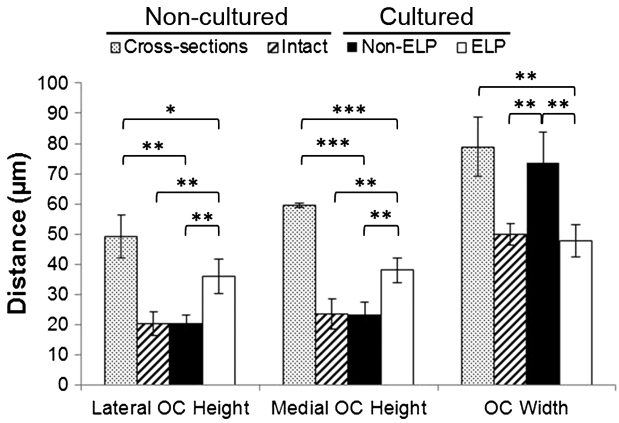

FIG. 5. Comparisons of lateral organ of Corti (OC) height, medial OC height, and OC width between cultured nonencapsulated (black bar) and cultured ELP-encapsulated cochlea (white bar) along a segment of the basal turn (3-day cultures). Comparisons are made to noncultured OC structure (striped bar) and noncultured cross-sections (dotted bar). Error bars represent SD (n = 3–4), *$p <$ 0.05, **$p < 0.01$, ***$p < 0.001$.

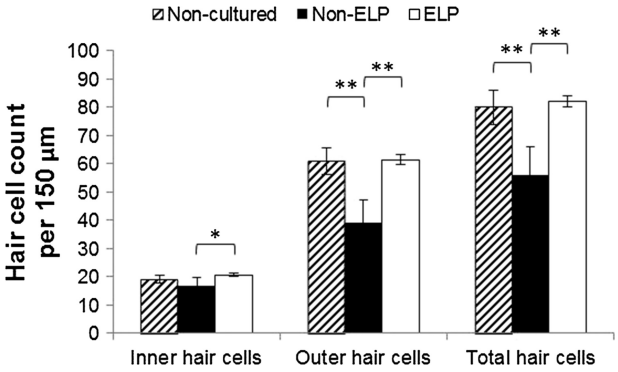

FIG. 6. Comparison of the number of inner hair cells, outer hair cells, and total hair cells per 150 μm along the basal turn for 3-day cultures. Comparisons are made to averages for noncultured P6 wild-type organ of Corti structure. Error bars represent SD (n = 3–4), $*p < 0.05$, $**p < 0.01$.

were also viable at 3 days (Fig. 7B). However, at 4 and 6 days of culture, nonviable cells were noted within the ELP-encapsulated cochlea (not shown).

Immunostaining of nonencapsulated cochlea and ELP-encapsulated cochlea showed no loss of MyosinVIIa-positive hair cells in either system at 3 days of culture. There was also no loss of MyosinVIIa-positive hair cells in the nonencapsulated system after 4 and 6 days of culture. Conversely, there was loss of MyosinVIIa-positive hair cells in ELP-encapsulated cochlea cultured for 4 and 6 days (not shown).

DISCUSSION

Organotypic cultures have been used for examining various aspects of cochlear physiology. The nonencapsulated control cultures described here are consistent with general methodology described in the literature (3,5,15,16). Cochlear tissues are placed on a substrate-coated flat surface and cultured in media for varying lengths of time. Although such a culture method allows various experimental approaches, it does not adequately maintain the cochlea's native structural integrity (15,16,18). These structural changes may alter the native cellular physiology and cell-cell interactions (40–42). Isolated organ of Corti cultured on a flat surface lacks the structural support provided by neighboring tissue in vivo to keep the organ in its native 3-D structure. As a result, cochlea culture studies have found the organ to flatten out when affixed to a flat surface (16,18,19). Structural integrity can also be lost if the cochlea floats off the adherent surface and becomes a floating or partially floating culture because these organs can fold onto itself (16).

In this study, we used an ELP hydrogel to encapsulate murine cochleae and provide structural support to aid in maintaining the desired native 3-D structure. This platform minimized the flattening and spreading of the organ of Corti that was otherwise observed in nonencapsulated cultured organs. Functional hair cell MET channels were also maintained within the encapsulated culture system. Cell viability was maintained for 3 days in culture; however, cell survival was compromised with longer culture duration. To our knowledge, this is the first report of the encapsulation and 3-D culture of the organ of Corti.

The changes in architecture of the cultured nonencapsulated cochlea demonstrated in this study are consistent with other reports (16,18,19). The nonencapsulated cochlea adhered to the coated coverslip began to lose structural organization by flattening and spreading out in culture. Comparison with measurements from noncultured cochlea showed conservation of structural integrity within the hydrogel.

Because of the diverse cellular environment and specific structural interactions that occur in vivo, maintaining the complex 3-D structure of the cochlea and organ of Corti in vitro is critical for maintaining proper function

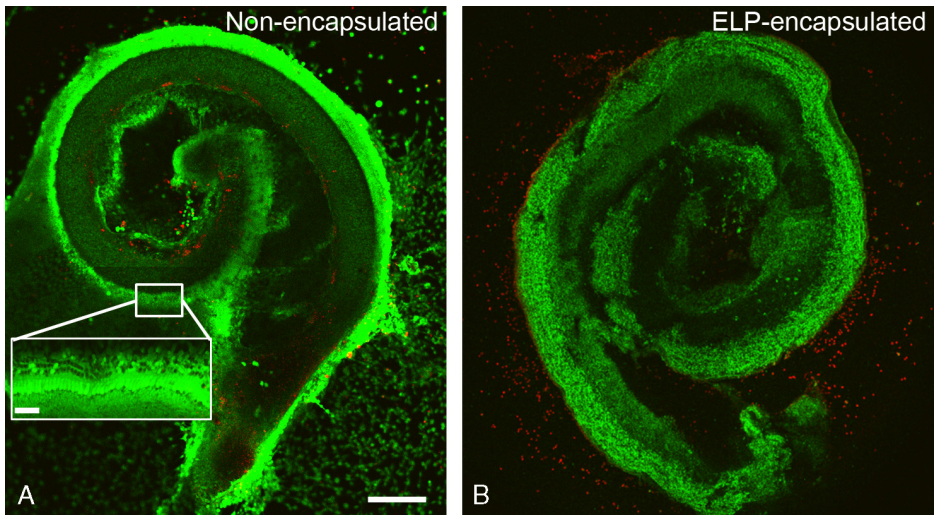

FIG. 7. Live/dead assay illustrating the viability in the *(A)* low- and high-magnification view of nonencapsulated cochlea and *(B)* low-magnification view of whole ELP-encapsulated cochlea after 3 days of culture. Live cells are labeled green and nuclei of dead cells labeled red. Scale bars = 200 μm in *A* and *B* and 100 μm in inset panel.

and more closely resembling in vivo biology (43). The ELP-encapsulated cochlea culture system successfully provided this 3-D environment for 3 days of culture and offers a tool to study various physiologic or pathologic conditions in vitro. Potential applications include the study of mechanisms regulating regeneration of hair cells, hair cell death caused by ototoxins, and cell migration in response to damage. At present, studies on these topics are mainly performed with cochlea cultured on substrate-coated flat surfaces and thus in conditions drastically different from the native cochlear environment (19,44). Outside the auditory system, 3-D cultures of hepatocytes and tumor cells have been used successfully and have shown improvement over 2-D cultures for drug screening (45–48). This 3-D culture system for cochlea can be similarly applied to investigating aminoglycoside toxicity (37,44).

Elastin-like protein hydrogels also have in vivo applications as they are biodegradable and have been successfully used as vehicles for drugs or gene delivery (49–51). Moreover, hydrogels can serve as a vehicle facilitating stem cell transplantation by providing a bioactive 3-D matrix. For instance, the hydrogel system has been shown to promote neurite outgrowth and chondrocytic differentiation (30,52).

A major limitation of the ELP hydrogel system involves the loss of cell viability beyond 3 days in culture, which may be caused by limited diffusion of nutrients. In addition, the complexities inherent to 3-D cultures provide several challenges including the requirement for a longer immunocytochemistry protocol for optimal staining (4 days compared with the 2-day protocol for nonencapsulated cultures). The ELP hydrogel used in this study provides an encapsulation material with tunable characteristics including concentration of ELP, percentage of cell-adhesive RGD sequence, as well as cross-linking density, which have a significant influence on cellular behavior (30,31). Variations in these material properties have yielded conditions that promote neurite growth in dorsal root ganglion cultures and regulated embryonic stem cell–derived cardiomyocyte contractility (30,31). Also, modifications of hydrogel properties can impact cell survival. Although increasing the total polymer concentration in poly(ethylene glycol) diacrylate (PEG-DA) hydrogels improves the compressive strength of the hydrogel, it adversely affects cell viability (53). Conversely, incorporating fibronectin-derived cell adhesion ligand RGDSP to PEG hydrogels causes a dose-dependent improvement in the viability of encapsulated cardiomyocytes (54). Similarly, addition of RGD sequences to PEG hydrogels enhanced the survival of encapsulated human mesenchymal stem cells in vitro (55). Together, these studies illustrate the extensive interactions between cells and their encapsulating biomaterials. Thus, future studies optimizing hydrogel properties may improve the applicability of hydrogel as a 3-D culture environment supporting the organ of Corti.

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