Published in final edited form as:

Biomaterials. 2008 March ; 29(8): 1028–1042. doi:10.1016/j.biomaterials.2007.11.006.

Peptide- and collagen-based hydrogel substrates for *in vitro* culture of chick cochleae

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Abstract

The overall goal of this work is to improve the culture of the auditory organ of birds for the dual use of developing a hair cell regeneration model and charting a pathway to the eventual replacement of the hearing organ. In doing so, we develop a protocol for removing the auditory organ from its basement membrane in the inner ear, attach the organ to a series of artificial basement membranes, and conduct qualitative and quantitative analysis of how cell morphology, viability and function change with time. Native matrix cultures, where the epithelium was floating in media with the basement membrane and accessory structures attached, were used as a basis of comparison. PuraMatrixTM, collagen I, collagen I/chondroitin-sulfate and MatrigelTM were chosen to encompass a diverse range of mechanical properties and macromolecule moieties. Surprisingly, we find that PuraMatrixTM outperformed the other matrices as a scaffold for sensory organ culture. PuraMatrixTM, a self-assembled peptide hydrogel, is a biochemically specific culture substrate that contains none of the extracellular matrix (ECM) molecules and growth factors contained in the inner ear's basement membrane. Rheological measurements reveal that PuraMatrixTM may be a closer approximation to the stiffness of the soft tissue supporting the auditory organ. Cell density on the PuraMatrixTM substrate is comparable to that of the native matrix cultures, despite the absence of the basement membrane and accessory structures. Further studies show that PuraMatrixTM supports the culture of functional hair cells over a 72 h period, with a significant increase in the number of functional hair cells in comparison to the organ cultured without a matrix. This is the first example of adhesion of the adult auditory epithelium to a biomaterial for an extended period of time. With further optimization, this system will enable the performance of many novel biophysical and pharmacological studies involving hair cells and supporting cells.

Keywords

Biomimetic material; Hydrogel; Organ culture; Cell morphology; Epithelium

1. Introduction

Auditory hair cells serve as the mechano-receptors for hearing, balance and orientation in space. Each hair cell is surrounded by several supporting cells and connects directly with the auditory nerve [1]. Hair cells are highly susceptible to noise, drugs and genetic defects, resulting

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in many cases of hearing loss. Unfortunately, hair cell loss in mammals is irreversible, and there is no cure [2]. Twenty years ago, it was discovered that hearing in the adult avian auditory organ regenerates spontaneously after noise and drug insults [3]. In the past 20 years, many *in vitro* and *in vivo* studies have been published that improve our knowledge of the molecular and cellular mechanisms involved in avian hair cell regeneration [4]. The avian auditory epithelium, known as the basilar papilla (Fig. 1), is anchored in the extracellular matrices (ECM) of the basilar and tectorial membranes, and flanked by cartilaginous plates. Hyaline cells connect hair cells to the inferior cartilaginous plate.

Hair cell regeneration in the chicken occurs along two pathways: proliferation of supporting cells followed by differentiation, and direct trans-differentiation of supporting cells [5]. Proliferation and trans-differentiation of mice neonatal supporting cells has also been observed, suggesting that supporting cell may be a target for therapeutic manipulation in humans [6]. Data from this study shows an average of 54 ± 25 Math1-GFP⁺ cells per culture differentiated from 500 supporting cells in a time span of 5 days *in vitro* [6]. Despite these advances, studies of extended adult organ explant culture have not been published. A prolonged explant culture model could enable *in vitro* studies of hair cell regeneration, and possibly even foment a pathway to functional organ replacement. The avian inner ear survives in the healthy bird and regenerates in response to damage *in vivo*. Given the proper conditions, the same should occur *in vitro*.

Adult avian inner ear explant cultures have typically been studied over a 72 h period [7–12], and studies of the adult mammalian inner ear in culture are often performed over the scale of minutes to a few hours [13]. To our knowledge, the avian inner ear has not been cultured with intact hair cell morphology for more than 3 days. The sensory epithelium has been cultured on an intact basement membrane in many previous studies. For example, free-floating, clotted well and plasma-clot culture paradigms were used, but the morphology of the organ had changed by 48 h [7]. Another study analyzed the effects of basic fibroblast growth factor on avian hair cell proliferation but only over a 48 h period [8]. The organ has also been placed on polyallomer rings glued to a coverglass with silicone tape, and the large-scale organization of the organ was preserved for 72 h [9]. The entire end organ has been adhered to Matrigel™. While improved in comparison to the no-Matrigel™ controls, hair cell density was significantly lower by 72 h [10]. The removal of the sensory epithelium from the native matrix and trituration of the tissue to isolate is an alternative method of culturing hair cells [11]. Hair cells have been cultured in this way for 10 days, but their identity within the organ becomes lost.

Clearly, success in understanding how the microenvironment affects hair cell regeneration and the movement towards organ replacement solutions are limited by our ability to culture the organ. These previous culture studies do not provide any insight into how the biochemical and mechanical influences of the surroundings regulate hair cell and supporting cell fate. To achieve insight, cell–cell contacts in the epithelium must be preserved while various substrates are used to study how the ECM affects the cells.

It has been shown in many systems that cell fate is affected by the local ECM. For example, the alveolar epithelial cell mesenchymal transition is thought to be regulated by the ECM [14]. In particular, cell viability in many systems has been shown to be influenced by the ECM. Human umbilical vein endothelial cell apoptosis was recently shown to be modulated by the small surface topology of an artificial ECM [12].

Evidence from *in vivo* and *in vitro* studies suggests that cell fate may be affected by the composition of the ECM *in vitro* in the chicken cochlea. The basement membrane underlying the avian sensory organ is known to be rich in laminin, collagen IV and II while lacking fibronectin [15,16]. The basement membrane underlying neighboring hyaline cells is rich in

fibronectin [15,16]. The fibronectin-rich matrix migrates with hyaline cells to take over part of the damaged epithelium in the events that follow noise trauma. Thus, the regulation of cell type and maintenance of a healthy epithelium may be regulated in part by the matrix underlying the cells. Moreover, in the chicken utricle, *in vitro* data suggests that cell shape and cytoskeletal conformation influence cell proliferation [17]. The outgrowth of the balance organ correlates with laminin and fibronectin coating concentrations when 200 $\mu\text{m} \times 200 \mu\text{m}$ pieces of the organ were cultured on polystyrene [17].

In this study, we adopt a protocol for isolation of the avian auditory organ [17] to culture the organ on four distinct artificial ECMs. In doing so, we test the hypothesis that anchoring the sensory epithelium of supporting cells and hair cells as a cell sheet on an artificial matrix will result in more effective hair cell cultures. In our analysis, we find that PuraMatrix™ (BD Biosciences, Franklin Lakes, NJ) is an advantageous culture substrate despite the absence of any of the ECM factors found in the native basement membrane.

2. Materials and methods

2.1. Sensory epithelium isolation and culture

The inner ears from 2-week-old chickens were dissected under sterile conditions immediately following sacrifice through an intraperitoneal injection of *Beuthanasia-D*. The otic capsules were exposed, and the heads placed in ethanol for 1 min. The organs were placed in ice-cold HEPES-buffered HBSS containing 120 $\mu\text{g/mL}$ AEBSF (Sigma) and 24 $\mu\text{g/mL}$ leupeptin (Sigma). The tegmentum vasculosa were removed. The organs were transferred to 1.5 mL of 0.5 mg/mL thermolysin [17] (Sigma) in HBSS, in the presence of 120 $\mu\text{g/mL}$ AEBSF and 25 $\mu\text{g/mL}$ leupeptin [18] at 37 °C for 22 min.

The organs were removed from thermolysin, and placed in artificial perilymph media, 37 °C, containing 137 mM NaCl, 5 mM KCl, 2.5 mM CaCl_2 , 1 mM MgCl_2 , 10 mM HEPES buffer, 2 mM sodium pyruvate, 2 mM creatine, 10 mM glucose, 11 mg/L Phenol Red and 25 mM NaHCO_3 . The tectorial membrane was removed with fine forceps, and the sensory epithelium isolated with a 26 3/8 gauge needle. The sensory epithelia were removed from the distal (low frequency) end to the mid-section of the organ.

Five milliliter of artificial perilymph was pipetted into the space beneath the surface of a transwell membrane (Corning Life Sciences, Corning NY, 3407: pore size = 0.4 μm). The artificial matrix was coated over the transwell membrane, and 1 mL of artificial perilymph was added to the surface. The epithelia were pipetted on the artificial matrix surface, hair cells facing up, with fine forceps. The transwell plate was placed in a LabNet (Edison, NJ) temperature controlled Mini-Incubator for 1 h at 37 °C, and 1 $\times$ N2 Hormone Supplement (Invitrogen Inc., Carlsbad, CA) was added to the wells. N2 was widely used in previous sensory organ culture studies [8–12]. After 48 h, the wells were placed in a six-well plate of fresh media, and 1 $\times$ N2 was again added.

The sensory epithelium dissection and isolation media were chosen to optimally remove the epithelium from the basement membrane with thermolysin while meeting the osmolarity and pH requirements and inhibiting endogenous protease inhibitor activity. In addition to osmolarity and pH regulation, the artificial perilymph system was chosen to mimic the ion concentrations of the native perilymph and meet the energy demands of the cells with creatine, glucose and pyruvate.

2.2. Artificial matrix preparation

2.2.1. Coverslips—Various sterile BD Biocoat™ (BD Biosciences, Franklin Lakes, NJ) coverslips were used. Sterile 12 mm poly-D-lysine, 12 mm laminin 12 mm poly-D-lysine/laminin

and 22 mm Fibronectin-treated BD Biocoat™ (BD Biosciences, Franklin Lakes, NJ) coverslips were placed in a 24-well tissue culture plate (Corning Life Sciences, Corning, NY). One milliliter of artificial perilymph media was added and the organ was placed on the coverslip, hair cells facing up.

2.2.2. PuraMatrix™—Five milliliter of artificial perilymph media was pipetted into the space beneath the surface of the transwell membrane. Thirty-eight microliter PuraMatrix™ (BD Biosciences, Franklin Lakes, NJ) was mixed with 112 μ L deionized water. The matrix was pipetted on the transwell membrane, adhering to the sides of the well. The matrix was left for 30 min to gel at 37 °C, 5% CO₂. One milliliter of artificial perilymph was added to the surface.

2.2.3. Collagen I Gel—Five milliliter of artificial perilymph was placed beneath the transwell membrane. Seventy-five microliter of bovine collagen I (BD Biosciences) was added to 15 μ L 10 $\times$ HBSS, 2 μ L NaOH and 58 μ L deionized water in a centrifuge tube on ice. The solution was mixed with the pipette, and 150 μ L was added to the transwell membrane's surface. The matrix was placed on the transwell membrane, adhering to the sides of the well. The matrix was given 30 min to gel at 37 °C, 5% CO₂, and 1 mL of artificial perilymph was added.

2.2.4. Collagen/chondroitin-6-sulfate—Fifteen milliliter of 0.53% w/v chondroitin-6-sulfate (BD Biosciences) in 0.05 M acetic acid was combined with 150 mL 0.5% w/v bovine collagen I (BD Biosciences) in acetic acid. The solution was sonicated and degassed, as previously described [19]. This stock solution was stored at 4 °C for up to 4 months. Five milliliter of artificial perilymph was added beneath the transwell insert. One hundred and fifty microliter of the collagen/chondroitin-6-sulfate solution formed a thick coating on the transwell plate after 30 min at 37 °C, 5% CO₂. One milliliter of artificial perilymph was added.

2.2.5. Matrigel™—Five milliliter of artificial perilymph was placed beneath the surface of the transwell membrane. Matrigel™ was thawed in ice, and 150 μ L was pipetted to the surface of the transwell membrane, adhering to the sides of the well. After a 30 min, 37 °C, 5% CO₂ incubation, the gel was washed with 20 $\times$ in 1 $\times$ HBSS until clear. Phenol red was removed from the gel. It is expected that gentamicin and growth factors were also dialyzed from the gel. One milliliter of artificial perilymph was added.

2.2.6. No matrix and native matrix controls—In the native matrix controls, the tegmentum vasculosum was removed from the inner ear organs, and the organs were placed directly in a 1 mL artificial perilymph solution on the transwell membrane, with 5 mL of media beneath the membrane. In the no matrix controls, the sensory epithelia were separated from the organs (see Section 2.1) and placed in the artificial perilymph solution, similar to the native matrix controls.

2.3. Immunohistochemistry

The samples were removed from the culture chamber, fixed in 2% paraformaldehyde for 15 min, permeabilized in 1% Triton, stained with 1:150 Alexafluor 488-conjugated Phalloidin (Invitrogen Inc., Carlsbad, CA) for 45 min, stained with 1:1500 To-Pro3 (Invitrogen Inc.), and mounted in VectaShield (Vector Laboratories, Burlingame, CA). Samples were washed twice in 1 $\times$ PBS between steps.

2.4. Live/dead assay

Ethidium bromide and Calcein AM (1:1000, Invitrogen Inc.) were added to the artificial perilymph. After 30 min, the organs were aspirated from solution in media, placed on slides, covered with coverslips and imaged at 20 $\times$ magnification.

2.5. Functional assay

FM1-43 is a fluorescently conjugated, small lipid molecule that penetrates the stereocilia bundles of functional cells [20]. FM1-43 fx (Invitrogen Inc.) is a fixable form of the molecule that was used as a marker for functional cells. The samples were placed in 10 $\mu\text{g/mL}$ FM1-43 fx [20], at room temperature, for 10 s, rinsed 1 $\times$ with 1 $\times$ HBSS for 10 s, and immediately fixed in 2% paraformaldehyde. The samples were then processed in the immunohistochemistry steps outlined in Section 2.3. Gadolinium, a mechano-transduction blocker, was used as a negative control [21]. In these experiments, the cells were incubated in 1 mM gadolinium chloride (Sigma-Aldrich) for 10 min at 37 $^{\circ}\text{C}$, prior to carrying out the functional assay. Finally, a “rescue” experiment was conducted in which the cells were incubated in gadolinium chloride for 10 min, washed with 1 $\times$ HBSS, placed in the FM1-43 dye, washed in HBSS and fixed. All concentrations and incubation times of this rescue experiment were the same as in the functional assay and the negative control.

2.6. Microscopy

Confocal scanning laser microscopy was performed on a Leica TCS SP confocal laser-scanning microscope (Leica Microsystems Heidelberg GmbH, Heidelberg, Germany). The digital images of compressed Z-series through sections of the basilar papilla of varying thickness were made using a 20 $\times$ (N.A. 0.7) or 40 $\times$ (N.A. 1.25–0.75) oil-immersion objective. The 20 $\times$ objective was used for the live/dead assay, and the 40 $\times$ objective was used for the functional and morphological studies. Smaller-scale morphological studies were carried out with the 40 $\times$ objective magnified by a 3 $\times$ zoom.

2.7. Cell count and density analysis

The cell density was compared in all of the samples. In doing so, six large squares within the epithelia were chosen in random segments dispersed along the length of the epithelium using the NIH Image J Toolbox, and stereocilia bundles within the squares were counted. The counts were divided by area to give density. Dissection-damaged regions of basilar papilla control samples were excluded from the analysis.

2.8. Functional assay analysis

The number of cells undergoing functional mechano-transduction was also estimated as a function of time in culture and culture condition. The percent difference in intensity of cellular labeling with FM1-43 fx vs. background in the gadolinium-blocked samples versus positive control samples was compared using the NIH Image J Toolbox. These values were used to establish a threshold for positive labeling. One to three 40 $\times$ images were taken per sample ($n = 2$ per condition), at random along the epithelia. Three images were used in the longer samples cultured on PuraMatrixTM, and one image per sample was used on the more condensed, circular free-floating samples. About 400 cells per image were present. The images were taken at random along the epithelia. In these images, every eighth cell was graded for functionality. The mean and standard deviation of the number of FM1-43-positive cells was reported as a function of culture condition.

2.9. Stiffness measurements

One milliliter of each material, as prepared in Section 2.1, was placed on a rheometer plate (AR 2000 Series Rheometer, TA Instruments, New Castle, DE). The bottom plate was set to 37 $^{\circ}\text{C}$, and the samples gelled over 30 min. A cone, 2 $^{\circ}$ taper was selected for the top plate. The frequency was varied, and stiffness was measured. The peak stiffness for each material at 1 Hz was recorded.

3. Results and discussion

3.1. Validation of sensory epithelium isolation procedure

The epithelia were removed from the basement membrane, from the distal end to the mid-proximal region of the auditory organ. The epithelia were about 1.5 mm long and 300 μ m wide. To verify that an intact sensory epithelium was removed from the basement membrane, a series of live/dead images were taken at 20 $\times$ magnification, after the isolation procedure ($n = 14$). About 1000 hair cells were present in each of these images. There were more than 50 dead cells on only one occasion (Fig. 2). Follow-up studies demonstrated that the hair cell bundles were morphologically closely packed following their removal from the epithelium by identifying the F-actin and nuclei distributions (Fig. 2). Finally, a series of z-stacks were acquired along the apical to basal plane of the epithelium to show two cell layers were present in the epithelium after the isolation from the inner ear (Fig. 2). Based on their anatomical positions, the two cell layers were concluded to be hair cells and supporting cells.

3.2. Sensory epithelium adhesion

The epithelia did not adhere to laminin, laminin/poly-D-lysine- and fibronectin-treated BD Biocoat™ TCPS coverslips. Epithelia did adhere to poly-D-lysine BD Biocoat™ coverslips. The epithelia also adhered to collagen I gel, collagen/chondroitin-sulfate, Matrigel™ and PuraMatrix™.

3.3. Stiffness measurements

Elastic stiffness measurements of collagen I gel, Matrigel™ and PuraMatrix™ were taken, and presented in a log/log plot (Fig. 3). The average stiffness of PuraMatrix™ at 1 Hz was 3 Pa, which is reasonable for this gel, whose weight is 99.5% water [22,23]. The stiffness of collagen I and Matrigel™ were in the 40–60 Pa range. The stiffness of collagen/chondroitin-sulfate was not obtained. The stiffness of the basement membrane has not been directly measured, but the stiffness of the gerbil basilar membrane has been estimated from measurements to be 9.1 Pa [24]. The elastic stiffness of all of these gels is much closer to the natural stiffness of the ECM surrounding the sensory epithelium than TCPS or glass.

3.4. Organ-level response to the substrates

The epithelia were cultured on each matrix for 24, 48, and 72 h (Table 1), and 40 $\times$ images of the F-actin-labeled ultra-structures were acquired (Fig. 4). The response of the epithelia as a function of time and condition is described in this section. The controls are the sensory epithelia cultured in free-floating conditions with their own matrices intact, denoted “native matrix” and with no matrix supporting them, denoted “no matrix” (Table 2). In some of the samples, the epithelium bent. This bending, or curling of the dissected organ is related to the release of prestress when it is removed from the surrounding tissues. Bending of the epithelium relative to the length of the organ, “longitudinal bending”, and relative to the z-plane, “radial bending”, were identified. The hair cell stereocilia bundles, which perform functional mechano-transduction, are referred to as “bundles”. The organs were removed from the anatomical distal (low frequency) end to the middle/proximal section of the tissue. The identification of superior, afferently innervated, versus inferior ends, efferently innervated ends [25] of the organ was noted: as was the distinction between low- and high-frequency ends. The distinction of the ends of the organ is important in distinguishing various cells at specific positions of the organ. There may be tonotopic differences in terms of cell behavior and microenvironmental regulation.

3.4.1. Cover slips—The epithelia did not adhere to laminin, laminin/poly-D-lysine- and fibronectin-treated BD Biocoat™ TCPS coverslips, but did adhere to poly-D-lysine BD

Biocoat™ coverslips. Tissue response to poly-D-lysine BD Biocoat™ coverslips was assessed. Extensive hair cell loss along the superior end was observed after just 24 h of culture on poly-D-lysine-treated BD Biocoat™ coverslips (Fig. 5).

3.4.2. PuraMatrix™—After 24 h in culture, the hair cell bundles were densely packed and round, as they are immediately following the removal of the sensory epithelium from the organ. This retention of hair cell density and morphology was an obvious improvement over culturing the epithelium on poly-D-lysine-coated coverslips. At the 48 h time point, a slight reduction in bundle density was observed. While the actin fibers of the stereocilia bundles were well grouped, the bundle orientations were more random, pointing in diagonal directions, in the 48 and 72 h samples. Some variation in the hair cell density was also noted in the 72 h samples. Patches of missing bundles appeared in two of the seven 72 h PuraMatrix™ samples, but many bundles were still observed in other areas of these two samples. The bundle density of the other five 72 h samples was slightly lower than at $t = 0$. A trend in hair cell density decline with location within the organ was not observed.

The low-frequency end of the organ was easily identified in every case except for one of the 72 h samples. Radial bending was observed in the low-frequency end in one of the 24 h samples, and along the entire length of the epithelium in two of the 48 h samples and in the longest four of the seven samples cultured for 72 h. Large longitudinal bends were observed in one of the four 48 h samples, and in four of the seven 72 h samples, particularly at later time points: indicative that in some samples the epithelium dislodged from the matrix during the culture process.

3.4.3. Matrigel™—After 24 h in culture, the bundles appear densely packed and round. All three of the epithelia cultured on Matrigel™ retained their length. By 48 h, a noticeable reduction in bundle density was seen independent of location within the organ in all of the samples. The bundles pointed in diagonal directions in the 48 and 72 h samples. The hair cell density varied greatly from sample-to-sample in the 72 h Matrigel™ samples, more so than the 72 h PuraMatrix™ samples. The density was noticeably but not significantly lower than optimal in three of the 72 h samples and dramatically lower in the other two, with large patches of missing bundles.

Longitudinal bending was noticed at the distal end of one of the 24 h Matrigel™ samples, and radial bending was observed at the distal end of two of the 24 h samples. Extreme longitudinal bending was observed in all of the 48 h samples, resulting in folds on the order of 400 μm . Three of the five 72 h samples were much shorter than the other two, possibly as a result of cracks in the gel. Radial bending was noticed in three of the 72 h samples. The distal end was easily discerned in all of the 24 and 48 h samples, and the elongated 72 h samples. Like in the PuraMatrix™ samples, the tissue bending and shrinkage that was commonly observed is likely a result of the release of the tissue from the substrate. Mechanical failure of the substrate may have also contributed.

3.4.4. Collagen/chondroitin-6-sulfate coating—After 24 h in culture, the bundles appeared densely packed and round, with a retained hair cell density. By 48 h in culture, a noticeable reduction in bundle density was seen in two of the samples, independent of location within the organ. The bundles were oriented in random directions in the 48 and 72 h organs. The density varied greatly in the 72 h samples, with two samples much less densely populated with hair cells than the other sample. In one of the samples, large areas without hair cell bundles were distributed along the length of the organ.

Longitudinal bending was seen in one of the 24 h samples. Folds on the order of 100 μm were present in two of the three 48 h samples, and in two of the three 72 h samples. The distal end

of the organ was easily distinguished from the proximal end in all of these cases. The folds were smaller than those observed in the Matrigel™ and PuraMatrix™ samples, and more likely to be a result of cell proliferation on a local scale than mechanical twisting of the epithelium on a global scale. Radial bending was observed in two of the four 48 h samples, most intensely in the distal end, and in two of the 72 h samples.

3.4.5. Collagen I gel—After 24 h in culture, the bundles appeared densely packed and round, and retained hair cell density. By 48 h in culture, a very large reduction in bundle density was observed, independent of location within the organ in two of the samples. The bundles were oriented in random directions and had elongated significantly. At 72 h, the bundles were oriented diagonally. A large drop in density was observed in all six samples.

The distal end was identified in all samples. The length of the epithelia was retained in all of the 24 h samples, with slight longitudinal bending at the distal end in two occasions. Radial bending was apparent in the distal end of one of the samples. Two of the three 48 h samples had an elongated morphology, and significant longitudinal bending was observed in a third sample. All six of the 72 h samples were also straight. A significant lateral shrinking was noted in two of the three 48 h samples, and five of the six 72 h samples. This shrinking occurred along the entire length of the organ. This shrinkage, coupled with the reduction of bundle density suggests the death of many hair cells in response to collagen I.

3.4.6. Native matrix—After 24 h in culture, the bundles appeared densely packed and round, and retained hair cell density. By 48 h, the bundle density varied slightly in the native matrix cultures. In one culture, a noticeable reduction in density was seen on the proximal tip of the sensory organ. In the second sample, this reduction was accompanied by a slight reduction in density on the superior edge, from the mid region to the proximal region. The orientation of the bundles was preserved. The hair cell bundles appeared diagonally oriented in the native matrix cultures after 72 h, but not as randomly oriented in all directions as they do in the 48 h matrix samples. The bundles are oriented more closely to the vertical axis than in the 48 matrix samples. There is a noticeable drop in hair cell density along the superior edge and at the proximal end of all of the samples. It appears that the cell density decrease progressed from more dense in the middle to less dense at superior end of the organ, with the exception of one sample, where no hair cells were observed in large areas in the mid-inferior and inferior regions of the organ. It is possible that cutting off the *in vivo* innervation to the cells resulted in reductions in hair cell number during the culture process. It was generally observed that more hair cells in the superior regions of the organ were lost. In particular, the tall hair cells, which receive mostly afferent innervation are located along the superior region of the organ [25]. The native matrix cultures retained their shape in culture all time points, allowing for an easy distinction of the high-frequency, neural and ab-neural regions.

3.4.7. No matrix—The bundles were very sparsely populated after 24 h in one of the samples that were cultured for 1 day. This sparse population may be a result of the epithelium being inadvertently turned over during the culturing or processing steps. The bundles all pointed in the same direction in the other two samples. In two of the three samples, 180° longitudinal bending and tissue overlap were observed, such that the low- and high-frequency ends of the organ could not be distinguished. The low-frequency end of the organ was identified in one of the three samples. By 48 h, the bundle density had decreased significantly. In two samples, the bundles were denser at the sample's exterior than in the interior. All epithelia were circular from longitudinal bending. The proximal, distal, superior and inferior sides of the epithelium could not be identified in these samples. Both of the samples were circular in shape at the 72 h time point. One sample had many observable hair cells, with bundles oriented diagonally. In the other sample, very few bundles were observed. It is likely that this sample was oriented

upside-down by mistake before the imaging took place. The distal end could not be distinguished in any of these samples.

3.5. Cell scale response

A follow-up study of hair cell bundle morphology as a function of culture time and matrix was conducted. Three images of the mid- to low-frequency section of the tissue halfway between the superior and inferior edges were acquired in each sample. Some trends were observed (Fig. 6). In all of the 24 h matrix samples, the hair cell bundles were arranged in round, tightly packed groups very similar to their arrangement before culture, while the bundles were diagonally oriented in all of the 48 and 72 h cultures. The bundles had also become longer and thinner by 72 h, particularly in the Matrigel™ and collagen I gel cultures. A similar bundle density decrease was observed in the native matrix controls, but the bundles better retained the wider, rounder appearance similar to the bundles in the 24 h time point. The orientations were closer to the *z*-axis in the 48 and 72 h native matrix samples than the 48 and 72 h matrix samples. In the no matrix samples, mid-low-frequency bundles could not be identified. A density drop-off was also observed in these samples, as were the diagonal bundle directions.

3.6. Hair cell density

The density (Fig. 7) of hair cells as a function of culture condition and time was quantified. It should be noted that hair cell density might favor the radially bent matrix samples slightly, if bundles were inadvertently double counted. However, the counts and densities of the upside-down no matrix samples were very low. Thus, the radial bend was not expected to add significantly to the densities of the matrix samples.

The densities of the 24 h samples were approximately 2.5 cells/100 μm^2 , mirroring the hair cell density immediately after the epithelium removal step (Fig. 7). The hair cell density dropped as a function of time. The PuraMatrix™ samples lost a significant density between 24 and 48 h ($p<0.05$). The Matrigel™ and native matrix samples had a retained density at 48 h but had significant decreases in hair cell densities by 72 h. The PuraMatrix™ cultures were significantly more dense than the no matrix cultures at the 24 h time point ($p<0.05$). The only case of one matrix performing significantly better than another is Matrigel™ performing better than collagen at the 48 h time point ($p<0.05$).

3.7. Live/dead assay

A live/dead assay was used to quantify how the cells responded to the microenvironment at 72 h of culture (Fig. 8). Consistently observed trends corroborated previous observations and suggested new findings. Again, the collagen I gel was the least ideal matrix. The sample widths had noticeably declined, and they were highlighted by many dead cells. More than 1.5 dead cells/100 μm^2 were observed on the collagen I gels, which is less than half of the initial hair cell density of 2.5 cells/100 μm^2 . About 1 dead cells/100 μm^2 was observed on the collagen/chondroitin-sulfate matrices. The collagen/chondroitin-sulfate samples were much larger on average than the collagen I samples (Fig. 9), as in the morphology study, where four of the five 72 h collagen I samples were very thin. With the exclusion of the thick outlier from the collagen I 72 h time point, collagen/chondroitin-sulfate supported significantly more hair cells for 72 h ($p<0.05$) than the collagen I gel.

The organs cultured on Matrigel™, PuraMatrix™ and without a matrix showed the best survival. PuraMatrix™ performed significantly better than both collagen I gel and the collagen/chondroitin-sulfate coating ($p<0.05$). Two of the Matrigel™ samples were longitudinally bent, while none of the PuraMatrix™ samples were longitudinally bent (Fig. 9). PuraMatrix™ allowed the organs to maintain their elongated morphology while preserving their viability in the culture environment.

PuraMatrix™ was determined to be the optimal matrix for hair cell culture due its specifically defined composition, softness and performance in terms of hair cell density, morphology and epithelium morphology.

3.8. Functional assay

FM1-43 fx was used as a marker to evaluate whether the placement of the organ on PuraMatrix™ promoted hair cell function. The no matrix condition was used as a negative control for the FM1-43 assay. FM1-43 fx enters into the hair cells that have an intact mechano-transduction apparatus in their stereocilia bundles [20]. Negative controls were conducted to show that brightly labeled hair cells were bright due to mechano-transduction, opposed to another means of entry. Gadolinium was used as a blocker of mechano-transduction ($n = 2$) [21], and compared with samples that were not treated with gadolinium and “rescue” samples which were washed between gadolinium and FM1-43 fx incubations. The positive control samples contained cells that were brightly labeled with FM1-43 (Fig. 10A), and other cells that were dimly labeled. The negative controls were brightly labeled in the membrane but not the cytoplasm (Fig. 10C) indicating that the uptake in the positive control samples was through the transduction apparatus. Some cells contained brightly labeled cytoplasm in the rescue condition (Fig. 10B). This data confirms that FM1-43 entered through the transduction channels of functional cells, and is reversibly blocked by gadolinium. Ten background samples were taken per image in each of these conditions, and the intensity of all cells were compared with background, using the Image J toolbox.

The intensity of cells in the negative control was 75 ± 19 (average $\pm$ standard deviation) percent greater than that of background ($p < 10^{-9}$). In the positive control the intensity was $187 \pm 52\%$ greater than background ($p < 10^{-25}$). In the rescue experiment it was $111 \pm 11\%$ greater than background. The high standard deviation was expected for the positive controls, where some but not all cells would be FM1-43 positive. A threshold of 135% difference between the cellular and background intensities was set to indicate functional cells.

Most of the cells were FM1-43 positive in the PuraMatrix™ and no matrix cases after 24 h in culture. The percentage of cells with bundles that also contained brightly labeled cytoplasm decreased with time in culture in both cases. In the later time points, it was evident that some cells had stereocilia bundles but were not labeled with FM1-43 (Fig. 10D). By $t = 72$ h, it appears that more cells with bundles co-labeled with FM1-43 when cultured on PuraMatrix™ than when floating in media. However, some hair cells were also functional when cultured without a matrix.

Our quantification of functional cells corroborated that a higher percentage of hair cells were functional when cultured on PuraMatrix™ than without a matrix after 72 h (Fig. 11) ($p < 0.05$). Also, a significantly greater number of cells were functional in all 24 h samples than 72 h samples ($p < 0.05$). In addition to keeping the cells anchored to a matrix, PuraMatrix™ yielded a higher percentage of functional cells.

3.9. Discussion

The sensory epithelium was removed from the basement membrane and cultured with its native matrix intact, with no matrix or on an artificial matrix. The live/dead assay confirms that the removal process did not result in a significant number of dead hair cells or supporting cells. In the artificial matrix cultures, the epithelium was anchored to a surface, with the supporting cells in contact with the matrix. A sheet of cells was obtained, as suggested by the presence of the expected tightly packed, crystalline-like array of hair cells at the 24 h time point (Fig. 6). The cells were cultured on the matrices without the influence of the many accessory cell types of the inner ear. The cultures with the epithelium on its native matrix were not fixed to a surface.

The gels outperformed the rigid coverslips in the epithelium adhesion and culture studies. The epithelium could be cultured on any of the four matrix substitutes over a 72 h period.

The sensory epithelia did not adhere to coated polystyrene coverslips. Warchol [17] observed that utricle epithelia pieces did adhere to laminin and fibronectin coated surfaces. This difference can be explained by sample size and shape. The utricle pieces were squares of 200 μm length, and the isolated epithelia in this study were about 1.5 mm by 300 μm . These sensory epithelium samples were both of a larger area and more oblong. More cells were required to establish connections with the artificial ECM, and the larger dimensions likely allowed for a greater possibility of epithelium bending (from the release of the physiological prestress upon dissection of the organ) inhibiting adhesion to the substrate. The organ was cultured in large segments to maintain the anatomical identity of the cells and avoid preferential damage to the peripheral cells of the smaller epithelia segments.

The epithelia adhered to poly-D-lysine but not ECM-coated coverslips. This difference can be explained by charge attraction. Poly-D-lysine-treated coverslips have a very high density of positive charges. Laminin and fibronectin are large glycoprotein macromolecules, with a lower charge density than poly-D-lysine and negatively instead of positively charged [28]. The negatively charged cell membrane experienced more attraction to the poly-D-lysine-treated surfaces.

The epithelia attached to the hydrogels, supporting cells facing down, much more favorably than to the coverslips. PuraMatrix™ is a repeating β -sheet [23], with the hydrophilic side exposed to the culture medium. The hydrophilic side contains alternating glutamic acid and lysine moieties, to provide positive and negative charges. Collagen fibrils consist of predominantly glycine repeats buffered by two amino acid moieties, often proline and hydroxyproline residues. Chondroitin-sulfate contains negatively charged carbohydrate groups. Growth factors were washed from Matrigel™, leaving predominantly collagen IV, laminin and entacin/nidogen. Entacin/nidogen is a sulfated glycoprotein with 5% carbohydrate content [28]. All of the hydrogels are charged.

The cellular response to the peptide- and collagen-based hydrogels was studied on cell and organ length scales, and as a function of time (Table 2). Hair cell density was the primary metric used to evaluate which matrix was optimal for culturing the cells. The hair cell density data shows retention of approximately 67% of hair cells on PuraMatrix™ and collagen/chondroitin-sulfate over 72 h, in comparison to roughly 50% in all other cases. 50% of hair cells were retained in the native matrix cultures, suggesting that removing the sensory epithelium from the basement membrane and anchoring it to an artificial matrix improved the hair cell cultures. PuraMatrix™ outperformed the native matrix cultures. PuraMatrix™ does not contain any ECM macromolecules. It is possible that defining the particular ECM macromolecules in the cochlea basement membrane and incorporating them into PuraMatrix™ would result in improved hair cell cultures. Matrigel™ did not yield any significant difference in hair cell number in comparison with PuraMatrix™, despite the fact that it contains many macromolecules known to be present in basement membranes. Of particular importance may be proteoglycans, which are known to promote tissue growth *in vitro* [27]. The similarity in density results between PuraMatrix™ and Matrigel™ may also suggest that soluble factors in the microenvironment may also be important in guiding cell fate.

In general, hair cell density was retained for 24 h, before declining between 24 and 72 h. The decline was approximately linear in the native matrix controls. The trends suggest that the decline in hair cell number was caused by shortfalls in the culture system of meeting the demands of hair cells, rather than by the trauma induced during the process of dissection. In our experience, if the latter were the major cause of the decline in hair cell density, one might

see more of a sharp decline in density over the first 24 h. The highly metabolic nature of hair cells may also play a role [29]. To meet this demand, media containing metabolic units such as glucose, ATP, pyruvate and creatine could be perfused past the hair cells. Tissue slices of other organs have benefited from perfusion *in vitro* [30].

It is unlikely in these experiments that supporting cells proliferated and trans-differentiated into hair cells during the 72 h incubations. Hair cell numbers declined as a function of time. Moreover, this activity is expected to take place after 72 h *in vitro*, as suggested by previous studies [6,31].

The data from the live/dead assay (Table 2) ruled out collagen I gel as the preferred culture matrix. A trend of hair cell death appearing in the proximal region of the organ first was evident in all but just two of the collagen I gel samples. This hair cell decline may be due to the use of collagen I in the culture system, instead of collagen II, which rests beneath supporting cells in the native matrix [16]. It may also suggest that the collagen I gel was excessively stiff (Table 2). The numbers of dead cells after 72 h are much lower on collagen/chondroitin-sulfate than on collagen I, suggesting that chondroitin-sulfate may have alleviated collagen I gel-mediated cell death. Chondroitin-sulfate may have signaled survival to the cells [27], or it is possible that the collagen/chondroitin-sulfate coating was more compliant [24], a further benefit to the cells.

In addition to hair cell density and the density of dead cells, bending was an important evaluation parameter (Table 2). In the no matrix condition, the epithelium bent severely both longitudinally and radially, to form a nearly spherical shape in three dimensions. In the native matrix case, however, bending was not observed. We postulate that since the organ is planar when it is intact in the body that the bending (or curling) occurs due to the release of prestress when the organ is dissected. The organ is attached to cartilaginous plates and hyaline cells along their length, and the basilar membrane at the base of supporting cells and the tectorial membrane at the apex of hair cells [25]. Hyaline cells are thought to play the role of stress fibers [16]. The role of tension in chicken hearing or hair cell regeneration is unknown. The importance of tension auditory function was recently suggested in a study of the gerbil cochlea [26].

Likely, the strength of adhesion of the organ to the substrate coupled with the mechanical properties of the substrate itself is at play in determining whether or not a dissected organ will bend. For example, if the adhesion is strong but the substrate gel is weak, then bending can still occur as the gel deforms or fails. It is also possible that the mechanism of the adhesion to the substrate is different along this heterogeneous organ, leading to variations in curling behavior between the different substrates. The collagen I gels were most effective in inhibiting the bending of the organ, but resulted a sharp decline in hair cell density, thinning of the samples and many dead hair cells in the live/dead assay. Matrigel™, on the other hand, did not inhibit the bending of the organ in spite of similar stiffness to the collagen I gels. It is possible that the epithelia did not remain adhered strongly to Matrigel™ during the duration of the culture process. Matrigel™ may have also reached a brittle failure in some cases. It could be interesting to repeat this experiment with Matrigel™ placed over a poly-D-lysine-treated coverslip, to see if the positive charge holds the gel in place and facilitate organ attachment to Matrigel™ over the 72 h culture period. Because collagen I gel was most effective in inhibiting the bending of the organs, it may also be beneficial to incorporate collagen I into a PuraMatrix™-based system for culturing the cells. Perhaps, the benefits of collagen I gel in bending-inhibition and PuraMatrix™ in promoting hair cell survival would result in an optimal culture system.

Another important observation was the lengthening of actin-based hair cell bundles (Fig. 1). The bundles are excessively long after 24 h in our culture system, suggesting that the cells did not properly regulate their lengths. Actin turnover is regulated *in vivo*, suggesting that an

element of the culture system was absent. Actin tread milling requires ATP [32], so the improper regulation of bundle lengths may be an indication of a need for improvements in meeting the tissue's metabolic demands. Bundle lengthening was most exaggerated in the collagen I gel and Matrigel™ samples, and observed to a lesser extent in the native matrix controls. The excessive bundle lengthening in the Matrigel™ samples in comparison with the native matrix samples may suggest deficiencies in the Matrigel™ matrix in recapitulating the native matrix. The absence of proper maintenance of bundle length with time was also observed in another study in which the organ, with the native matrix intact, was cultured on Matrigel™ [10]. This evidence may also suggest the importance of catering the soluble microenvironment to meet the metabolic demands of the cells.

Collectively, this data suggests that PuraMatrix™ was the best scaffold for adult avian sensory epithelium cultures. Hair cell density was well retained over 72 h. Dead cell density was very low at the 72 h time point. The epithelia were elongated, which allows for the study of differences in cell behavior as a function of location in the organ. The organs were entirely free of hair cell-less patches. There was much more variability in the response to Matrigel™, and many dead cells on collagen/chondroitin-sulfate and collagen I gels.

An FM1-43 fx assay was run with PuraMatrix™ cultures to determine whether there was a link between the presence of a matrix resting beneath the cells and an intact functional mechano-transduction apparatus (Fig. 1). More cells were positive for FM1-43 when cultured on a matrix than when cultured without a matrix. Thus, PuraMatrix™ supported a dense epithelium of viable and functional hair cells for 72 h. This observation, in conjunction with the observation of very few dead cells in the no matrix controls after 72 h, suggest that while a matrix is not necessary for the culture of viable hair cells, it improves in the culture of functional hair cells. The increased uptake of FM1-43 may be a result of more intact cell-cell arrangement in the anchored, in comparison with floating condition. Extreme longitudinal bending was observed in the no matrix controls. This bending may have been accompanied by rearrangement that resulted in a loss of function.

About 60% of the hair cells were FM1-43 positive after 72 h when cultured on PuraMatrix™. Thus, there is room for improvement in tailoring the microenvironment to meet the functional demands of hair cells. For example, it may be possible for the epithelia to be cultured with the presence of an ionic gradient and a standing current of potassium [33,34]. Functional mechano-transduction is accompanied by a potassium influx. The ionic gradient would enable the influx of potassium into the cells. The standing current would restore the potassium gradient for the next cycle. Moreover, there may be a link between viability and functional mechano-transduction. It has been shown that high potassium concentrations attenuate ischemia-induced hearing loss [34]. Moreover, normal gap junction function is required for potassium cycling and restoration of the trans-epithelium ionic gradient [3,33]. Knockouts for Connexin cause a loss of hearing *in vivo* [3] through the inhibition of this cycle.

PuraMatrix™ is a chemically defined, soft substrate that supports hair cell cultures for 72 h *in vitro*. PuraMatrix™ outperformed native matrix cultures, in which all accessory structures were intact. Improvements to the culture system can be made, however. Hair cell density declined with time, slight epithelium bending was observed in some cases and the proper bundle lengths were not maintained over the culture duration. The incorporation of glycosaminoglycan units may promote epithelium growth. However, the native matrix should first be fully characterized to determine what macromolecules are present. The loss of hair cells and proper bundle regulation was observed even in the native matrix cultures. The perfusion of media, or the implementation of a trans-epithelium ionic gradient *in vitro* may better meet the metabolic and functional demands of the hair cells.

4. Conclusions

In this study, we detached the avian sensory epithelium from its basement membrane and attached it, with supporting cells facing down, over four different collagen- and peptide-based artificial matrices. Hair cell density was observed in response to these matrices after 24, 48 and 72 h. Dead cell density was observed after 72 h. The peptide-based matrices, PuraMatrix™ and Matrigel™, outperformed the collagen-based matrices by these metrics. A higher cell density was found on PuraMatrix™ than cultures in which the epithelium was attached to its native matrix after 72 h. This suggests that detaching the epithelium from its native matrix and anchoring it to an artificial matrix is the preferred method of culturing the cells.

Qualitative phenomena such as epithelium bending and hair cell bundle length were followed, to discern the differences in tissue response to PuraMatrix™ and Matrigel™. Very little bending was prominent on the PuraMatrix™ cultures, and much more bending was prominent on Matrigel™ cultures. The distal end was easily identified in the PuraMatrix™ cultures, enabling the correlation to be made between microenvironment the fate of individual cells within the organ. The hair cell bundles maintained their round form for 24 h, before taking on a more elongated appearance and diagonal direction of orientation. Many of these cells were functional at the 72 h time point when cultured on PuraMatrix™ as suggested by the FM1-43 assay.

Use of PuraMatrix™ resulted in higher cell density, more normal bundle morphology and more functional cells at the 72 h time point. Experiments requiring functional hair cells up to 3 days would benefit from the use of this new culture protocol. The identification of the distal end, combined with the maintenance of an elongated structure, with a high density of hair cells and low density of dead cells suggest that with further optimization this setup is suitable for the performance of many novel physiological and pathophysiological assays on hair cells. The techniques established here lay the early foundation for the development of an *in vitro* hair cell regeneration model and a small step along the pathway to functional replacement of the hearing organ.

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Fig. 1.

The adult avian auditory epithelium. Hair cells are labeled with F-actin marker Phalloidin. Hair cells in the superior (S) region receive both afferent and efferent innervation, while the inferior (I) hair cells receive predominantly efferent innervation. Low-frequency hearing occurs at the distal (D) end of the organ, while high-frequency hearing occurs at the proximal (P) end.

Fig. 2. Confirmation of auditory epithelium isolation. A plane of Phalloidin-labeled hair cells with round bundles was isolated (left). Z-series of Phalloidin and nuclear labeling confirms that two cell layers were isolated (left). This procedure resulted in very little cell death in the live/dead assay (right).

Fig. 3.

Log/log plot of elastic stiffness (G') measurements of the hydrogels, as a function of angular frequency. The values were recorded at an angular frequency of 1 rad/s (circled). The elastic stiffness (G'') of PuraMatrix at 1 rad/s was on the order of 1 Pa, while the elastic stiffness of Matrigel was on the order of 50 Pa. The mean elastic stiffness of Collagen I gel was also on the order, but the Collagen I gel stiffness measurements were non-repeatable.

Fig. 4. Phalloidin-labeled sensory epithelia after being cultured on the matrices for 24, 48 and 72 h and fixed. Of the four matrices, the least bending was observed on PuraMatrix. Extensive hair cell loss is observed on the collagen I gels.

Fig. 5. Phalloidin-labeled sensory epithelium after 24 h of culture on poly-D-lysine-treated coverslips. Extensive hair cell loss was observed.

Fig. 6. High-magnification comparison of F-actin-labeled hair cell bundle morphology. Elongated and diagonal hair cell bundles were observed beyond 24 h when the organs were cultured in all conditions.

Fig. 7. Quantification of hair cell density as a function of time and culture condition (data displayed as mean and standard deviation).

Fig. 8. Quantification of dead cells present on the matrices after 72 h (mean and standard deviation density).

Fig. 9.
Visualization of live (green) and dead (red) cells after 72 h in culture.

Fig. 10.

Visualization of hair cell FM1-43 fx distribution. FM1-43 fx enters the transduction channels of hair cells (A). This entry can be rescued by a wash in $1 \times$ HBSS (B) following inhibition by the transduction-channel blocker gadolinium (C). Some but not all hair cells were functional after 48 h in culture on PuraMatrix (D).

Fig. 11.
Number of FM1-43 fx-positive cells as a function of time and culture condition (mean and standard deviation).

Table 1
Experimental design: summary of the number of epithelium samples cultured for each time point and culture condition

Condition (h)	PuraMatrix (N)	Collagen/chondroitin-sulfate	Collagen I gel	Matrigel	Native matrix	No matrix
24	5	2	3	3	2	3
48	5	3	3	3	2	3
72	7	3	3	6	3	2

Table 2

Data summary of how output parameters hair cell density, dead cell density, epithelium bending and hair cell bundle morphology, and FM1-43 fx labeling changed as a function of matrix and stiffness

Culture condition	Elastic stiffness (Pa)	Hair cell density (per 100 μm^2)			Dead cell density (per 100 μm^2) 72 h	Bending phenomena (longitudinal; radial)	Bundle morphology (shape; length)			Percent FM1 -43 positive 72 h
		24 h	48 h	72 h			24 h	48 h	72 h	
PuraMatrix	2.3 $\pm$ 1.4	2.4 $\pm$ 0.5	1.7 $\pm$ 0.5	1.6 $\pm$ 0.5	0.2 $\pm$ 0.3	Mild, moderate	Round, compact	Round, long	Sharp, long	66 $\pm$ 2%
Matrigel	56 $\pm$ 6	1.9 $\pm$ 0.1	2.3 $\pm$ 0.4	0.9 $\pm$ 0.6	0.0 $\pm$ 0.0	Moderate, moderate	Round, compact	Round, long	Sharp, long	N/A
Collagen I gel	44 $\pm$ 39	2.4 $\pm$ 0.3	1.2 $\pm$ 0.5	1.2 $\pm$ 0.6	1.7 $\pm$ 0.9	Very mild, very mild	Round, compact	Sharp, very long	Sharp, very long	N/A
collagen/chondroitin-sulfate	N/A	2.0 $\pm$ 0.6	1.6 $\pm$ 0.44	1.5 $\pm$ 0.2	0.8 $\pm$ 0.3	Mild, moderate	Round, compact	Round, long	Sharp, long	N/A
no matrix	N/A	1.1 $\pm$ 0.2	1.6 $\pm$ 0.2	0.8 $\pm$ 0.5	0.1 $\pm$ 0.0	Severe, severe	Round, compact	Round, long	Sharp, long	33 $\pm$ 16%
native matrix	10	2.2 $\pm$ 0.2	1.6 $\pm$ 0.1	0.9 $\pm$ 0.3	N/A	None, none	Round, compact	Round, long	Round, long	N/A