Supporting Information:

A 3D Printed Bionic Ear

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Materials and Methods:

Chondrocyte Culturing

Chondrocytes isolated from the articular cartilage of one month old calves were obtained from Astarte Biologics (Redmond, WA). The cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) with 10% fetal bovine serum for 6 to 8 days. A 1% antibiotic-antimycotic solution consisting of 10,000 U/mL penicillin G sodium, 10,000 μg/mL streptomycin sulfate, and 25 μg/mL amphotericin B in 0.85% saline was also added to prevent contamination. The cells were cultured at 37 °C and 5% CO_2.[1] Once the initial seeding density of the cells was reached, the viability was determined using a Trypan blue (Corning Cellgrow, Mediatech, VA) cell exclusion assay. The chondrocytes were diluted in acid azo exclusion medium of the dye into a 1:1 solution of the cell suspension in 0.4% Trypan blue dye. The cells were incubated in the medium for less than 5 minutes. The nonviable cells that stained blue were then counted under a microscope and found to be 96.58 ± 1.64%. The chondrocytes were then suspended in phosphate buffered saline and pelleted by centrifugation.

Alginate Formulation and Chondrocyte Seeding

To make the hydrogel matrix, low-viscosity, high G-content non-medical grade alginate protanal LF10/60 alginate (FMC Biopolymer, Drammen, Norway) was dissolved at a concentration of 30 mg/mL, removed of clumps by passing through a 0.22 μm filter, and mixed with the cell pellet by gentle stirring. The alginate-cell suspension was vortexed and mixed in a 2:1 ratio with autoclaved 5 mg/mL $CaSO_4$ in PBS to achieve the desired final cell seeding density (60×10^6 cells/mL for the printed ears and 20×10^6 cells/mL for comparison of mechanical properties).

3D Printing

A CAD file of the bionic ear in STL format was used to define the print paths by slicing the model into layers of contour paths and raster fill paths. Each of the functional materials used for the creation of the bionic ear, including cells, conducting polymers (Silicone Solutions, Twinsburg, OH), and structural polymers (RTV silicone, 3M, St. Paul, MN) were then loaded into the deposition tool and printed in the spatial heterogeneity determined by the CAD.

The efficiency of the 3D printing process in printing the biological cells was assessed by comparing the viability of the chondrocytes before and after the printing process. The efficiency of the 3D printing process in printing the electronic material was characterized by comparing the resistivity of the printed coil geometry at various volumetric flow rates (Fig. S1). To aid the comparison, 'print efficiency' was arbitrarily defined as the ratio of the theoretical resistivity of the material to the resistivity of the 3D printed material. Resistivity was measured using a 4 point probe apparatus to negate contact resistance. Print efficiencies for various printing speeds were calculated and compared to identify the optimum printing conditions. The 3D printed bionic ear was then cultured in the same medium as above containing 10% or 20% fetal bovine serum (FBS) at 37 °C and 5% CO_2 (Figs. S2 and S3). The chondrocyte to feed medium ratio was kept below 1.7 million cells/mL per day to ensure sufficient nutritional supply to the cells. The gross morphology of the bionic ear after 10 weeks of *in vitro* culture is shown in Fig. S4.

Seeding chondrocytes into a bioabsorbable alginate matrix and shaping it via 3D printing localizes the cells to a desired geometry, allowing for new ECM production in defined locations when cultured in nutritive media. To evaluate the degradation of the alginate scaffold under culture, we compared the mean weights of the printed ear constructs consisting only of alginate to those of constructs printed with chondrocyte-seeded alginate. As the cartilage tissue develops

in the cellular constructs, the polymer scaffold is slowly reabsorbed (Fig. 2D), so that new tissue retains the shape of the polymer in which the cells were seeded. The mean weights of the alginate-only constructs were found to decrease over time in culture, indicative of the slow degradation of the hydrogel matrix. The biodegradable scaffolding provides each cell with better access to nutrients and more efficient waste removal.

To demonstrate the versatility of our approach in modifying the final organ by modifying the CAD design, we printed a complementary left ear by simply reflecting the original model and following the same culturing conditions as before (Fig. S5). The media after incubation was tested for bacterial contamination with 100 μM BacLight Green stain (Molecular Probes, Eugene, OR). To verify the effect of the culturing on the electronic properties of the AgNP-infused conducting silicone, the resistance of the coil antenna and the cochlear-like electrodes were measured at various points in culture using a 4 point probe measurement apparatus. The resistance was found to be constant over time in culture, because the silicone covering the outer surface of the conducting traces forms an insulating and water-proof coating. This was confirmed via SEM micrographs of the 3D printed conducting traces (Fig. S6).

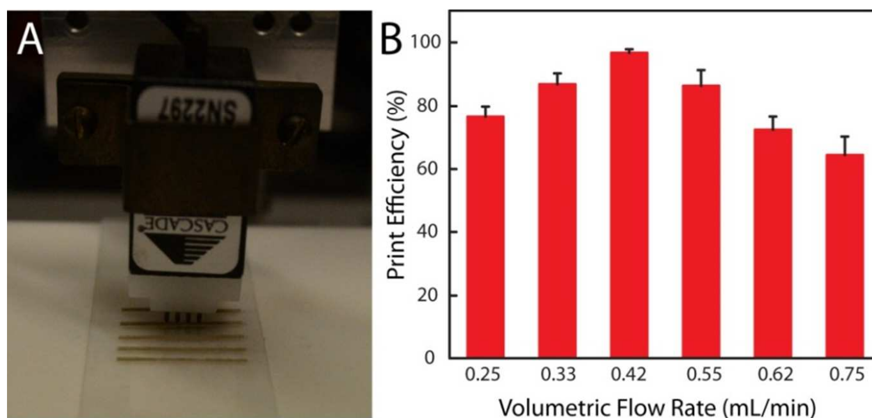

Figure S1. Resistivity measurements. (**A**) Image of the four point probe measurement of conducting traces of AgNP-infused silicone printed at various volumetric flow rates. (**B**) Print efficiency at various volumetric flow rates. Error bars show standard deviation with N=3.

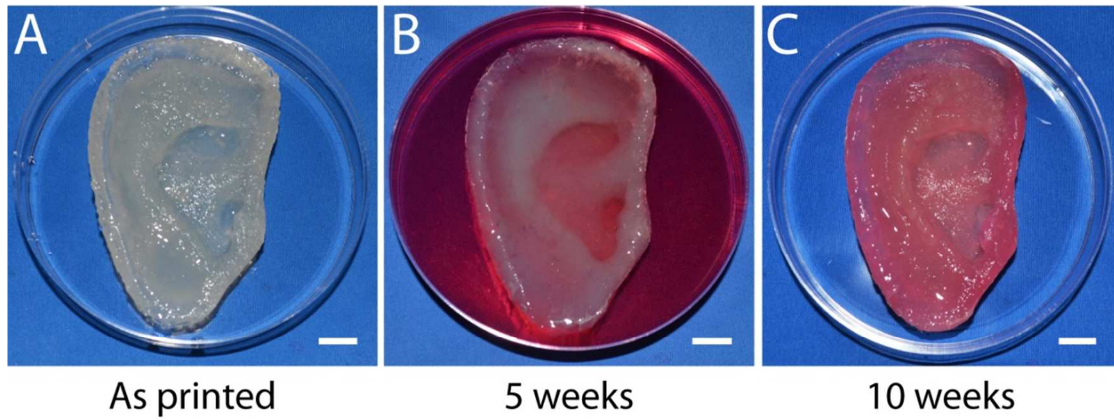

Figure S2. Images of the 3D printed ear auricle cultured in 10% FBS at various stages of growth. (**A**) As printed, (**B**) after 5 weeks in culture, and (**C**) after 10 weeks in culture. Scale bars are 1 cm.

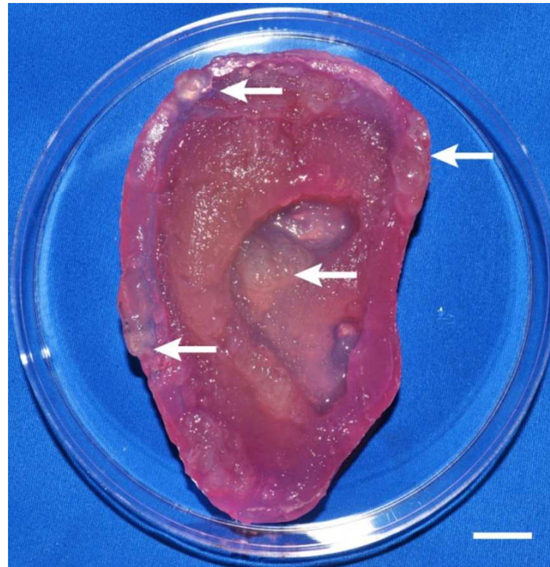

Figure S3. Image of neocartilage growth of the 3D printed ear under culture containing 20% FBS, showing bulbous outgrowth on the surface as indicated by the arrows. Scale bar is 1 cm.

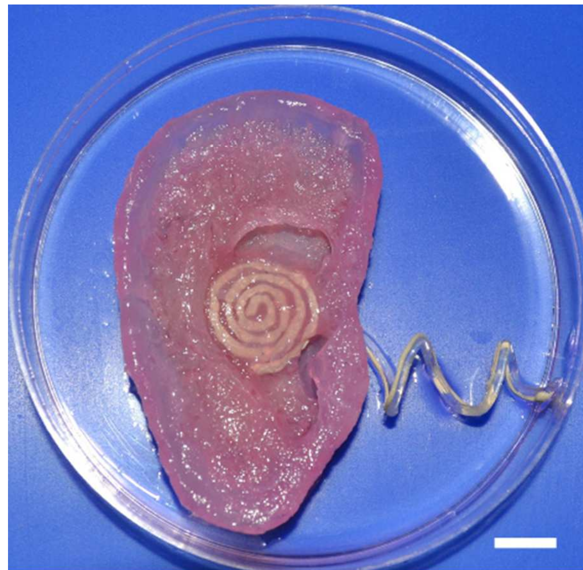

Figure S4. Gross morphology of the 3D printed bionic ear after 10 weeks of *in vitro* culture. Scale bar is 1 cm.

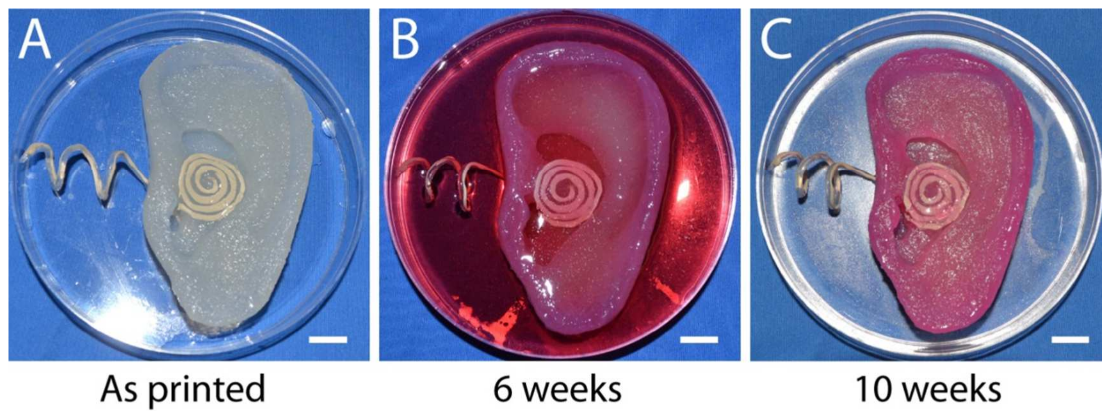

Figure S5. Images of the 3D printed left bionic ears at various stages of growth. (**A**) As printed, (**B**) after 6 weeks in culture, and (**C**) after 10 weeks in culture. Scale bars are 1 cm.

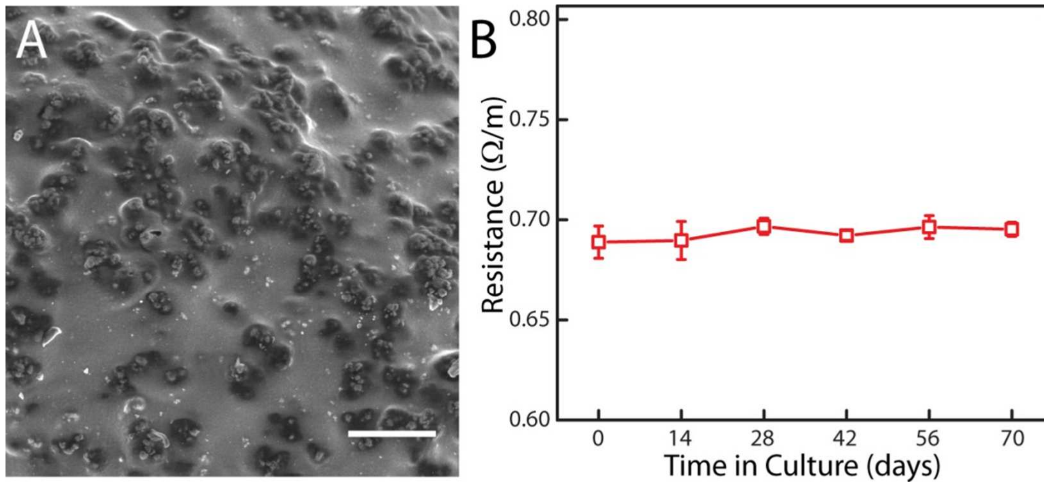

Figure S6. Electrical resistance of the coil antenna in culture. (**A**) SEM image of the coil antenna surface. Scale bar is 100 μm. (**B**) Resistance per unit length of the coil antenna over time in culture. Error bars show standard deviation with N=3.

Cellular and Tissue Viability

The effect of the 3D printing process on the viability of the chondrocytes was analyzed by measuring the viability before and immediately after the printing process (Fig. S7). First, the viability of the chondrocytes after mixing with the alginate hydrogel was tested with a LIVE/DEAD® Viability Assay (Molecular Probes, Eugene, OR). The samples were stained with 0.15 μM calcein AM and 2 μM ethidium homodimer-1 (EthD-1) for approximately an hour at room temperature. The viability of the cells in the printed ear construct was also measured by taking specimens from various locations. The stained samples were analyzed under a fluorescent microscope (Olympus BX60) with dual band (FITC-Texas red) filter and the viability was calculated as the average of the ratios of live over total cells in a given field.

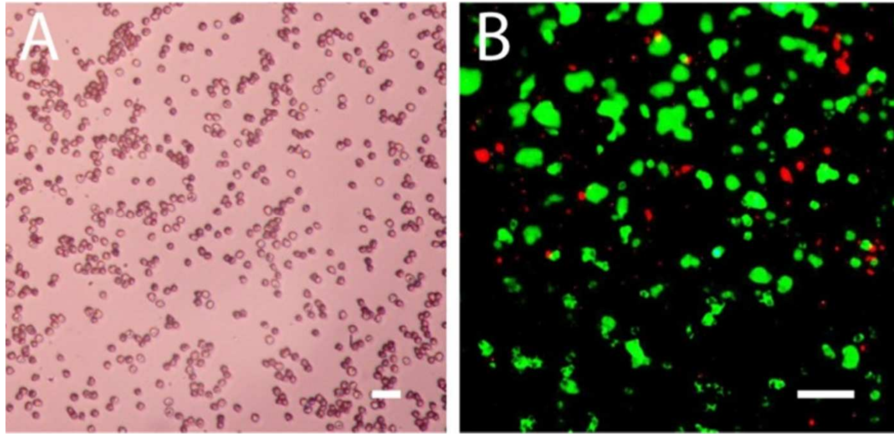

Figure S7. LIVE/DEAD® assay of chondrocytes. (**A**) Trypan Blue exclusion assay and (**B**) calcein AM and ethidium homodimer-1 LIVE/DEAD® assay immediately after 3D printing. Scale bars are 50 μm.

Tissue level viability of the bionic ear was assessed using fluorescent staining at various sites in the neocartilage tissue of the ear. Specifically, the chondrocytes were stained with 2 μg/mL fluorescein diacetate (FDA) and 0.1 mg/mL propidium iodide (PI). The viability of the cartilage tissue that was in contact with the coil antenna was examined at various locations. To examine the viability of the tissue at the interface of the cartilage tissue and the electrode, a cross section of the ear was taken and assay was performed on the cells that were in contact with the electrode.

Biochemical Analyses

Biochemical analysis was performed on the 3D printed ear under various stages of culture to determine the cell proliferation and ECM characterization. Samples were removed from the ear under culture, weighed and kept frozen. Samples were then digested in 1 mL of papain digest buffer (0.1 M sodium phosphate, 10 mM sodium EDTA, 10 mM cysteine hydrochloride, and 3.8 U/mL papain, all from Sigma Aldrich, St. Louis, MO) at 65 °C for 24 h.

Chondrocyte proliferation in the bionic ear under culture was determined by measuring the DNA content of the samples. In short, the DNA content was quantified by measuring the amount of fluorescence (358/458 nm) after exposing to Hoechst 33258 dye.[2] To convert the obtained fluorescence from the samples to a quantified value in terms of weight, the fluorescence was compared with a standard curve created with calf thymus DNA (Fig. S8). Hoechst 33258 dye was kept as a stock solution of 1 mg/mL in distilled water and stored in a foil-wrapped container at 4 °C. A working solution was diluted to 0.1 µg/mL in 10 mM Tris, 1 mM Na EDTA, 0.1 mM NaCl, pH 7.4, immediately before use and was dispensed by minimal exposure to light. Calf thymus DNA was made to 100 µg/ml in PBS and stored frozen. Next, the emission and excitation spectra (358/458 nm) were measured for the Hoechst 33258 dye alone and in the presence of calf thymus DNA and papain digested chondrocytes from the samples (Fig. S9).

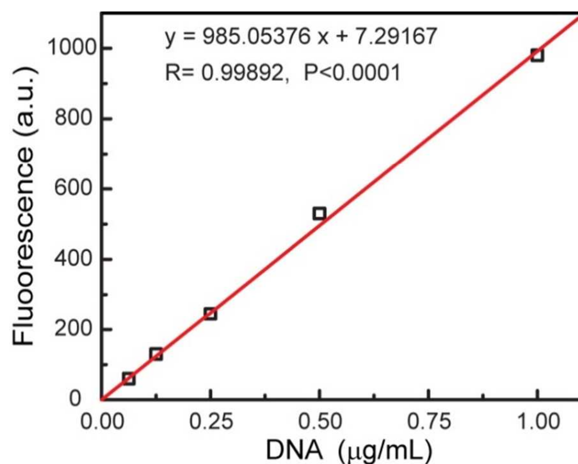

Figure S8. DNA content standard curve obtained from calf thymus DNA.

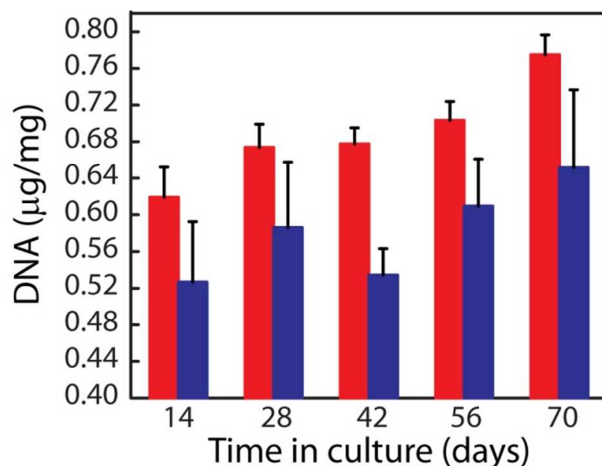

Figure S9. DNA content in the 3D printed ear at various stages during culture with 20% FBS (red) and 10% FBS (blue). Error bars show standard deviation with N=3.

Analysis of the contents of the extracellular matrix (ECM) was performed to evaluate the metabolic profile of the 3D printed ear under culture. The amount of collagen content – a major component of the extracellular matrix secreted by the chondrocytes under culture – was determined from the measurement of HYP in the digest.[3] The amount of HYP in the sample was determined from the absorbance measured using a standard curve created from L-Hydroxyproline (Sigma Aldrich) (Fig. S10). The samples were first hydrolyzed in 6 N HCl at 110 ºC for 18 h in a test tube. The volume of the sample containing an estimate of 0.2-6 μg HYP was brought to 2 mL. The pH of the solution was also adjusted to the pH of chloramine-T reagent. The 2 mL sample was then mixed in a test tube with 1 mL chloramine-T solution both having a temperature of about 20 ºC and kept for about 15 minutes. 1 mL of the aldehyde/perchloric acid solution was then added and mixed thoroughly. The temperature of the sample was then kept at 60 ºC by immersing in to a hot water bath for about 20 minutes. The test tube was then cooled to room temperature under running water and absorbance was detected at 560 nm with a spectrophotometer.

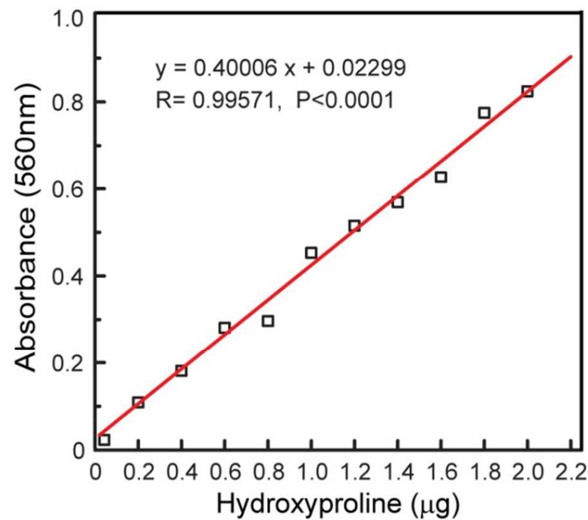

Figure S10. Hydroxyproline standard curve obtained from L-Hydroxyproline.

The proteoglycan content in the ECM was evaluated by the quantification of the total sulfated GAGs in the sample. The GAG assay was performed by the spectrophotometric detection of the chromatic changes that occur when 1,9-dimethylmethyline blue (DMB), a cationic dye, binds to the sulfate and carboxyl groups present in the GAG chain. To eliminate interference from the binding of DMB with the carboxyl groups in the alginate, the assay was performed at a pH of 1.5, which has been shown to block the effect from the alginate. Stable solutions of the DMB dye were prepared according to standard protocols [4] and mixed with the sample digest, and the absorbance at 595 nm was read. A standard curve was created using Chondroitin-6-Sulfate (C-6-S) from shark cartilage (Sigma Aldrich) and used to obtain the quantitative values of the GAG content from the observed absorbance (Fig. S11).

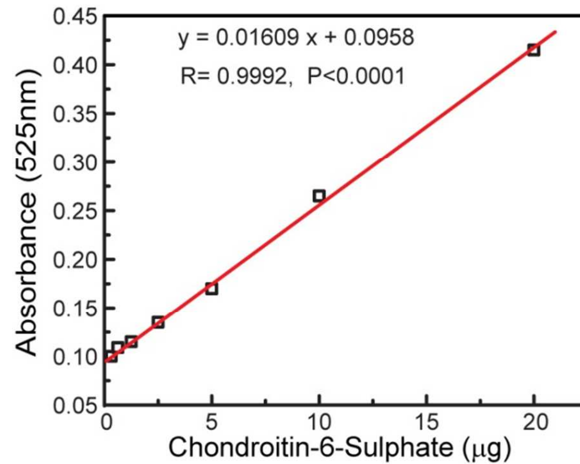

Figure S11. GAG standard curve obtained from Chondroitin-6-Sulphate.

Histologic Evaluation of the Bionic Ear

We performed basic histological analysis for the general assessment of cell and tissue morphology and distribution using the hematoxylin-eosin stain.[5] For the histologic evaluation of the cartilage tissue formed in the bionic ear under culture, specimens were removed from the ear after 6 weeks and 10 weeks of *in vitro* culture and kept frozen. The specimens were then fixed in 10% unbuffered formalin supplemented to 0.1 M in $CaCl_2$ for about 24 hours and then embedded in paraffin. The specimens were coarsely sectioned using a razor blade, followed by a microtome. For hematoxylin-eosin staining, the sections were first rinsed with 3 changes of 5 minutes in xylene followed by rinsing in 95% ethanol for 2 minutes. The sections were then immersed in Mayer's hematoxylin solution (1 g potassium ammonium, 1 g Hematoxylin, 0.2 g sodium iodide and 1 g citric acid in 1000 mL distilled water) for 10 minutes. The sections taken out of the staining solution were rinsed in running tap water and in 95% ethanol. The sections were then immersed in 0.25% Eosin Y solution (250 mL of Eosin Y stock solution and 5 mL of

glacial acetic acid in 800 mL of 80% ethanol) for a minute followed by 3 changes in 100% alcohol. Finally, the sections were mounted on a glass slide and rinsed with xylene.

The sections were also stained with Safranin O to visualize the amount of proteoglycan content. The sections were deparaffinized and hydrated using distilled water followed by immersing in Weigert's Iron Hematoxylin for 5 minutes. The sections were washed gently in distilled water few times until the excess dye is removed. The tissue was then differentiated in 1% acid- alcohol (100 mL of 70% ethanol and 1 mL of glacial acetic acid) solution for 5 seconds and rinsed thoroughly in distilled water. The sections were subsequently immersed in 0.02% Fast green (0.05 g of Fast green in 25 mL distilled water) for approximately 1 minute, followed by 1% acetic acid for 30 seconds. The staining was completed via immersing in 1% Safranin O (2.5g Safranin O in 250 mL of distilled water) for 10 minutes. The sections were then gently rinsed in 95% ethanol and slowly dehydrated by 2 changes in 95% ethanol and 2 changes in 100% ethanol. The stained sections were mounted on cover slips and rinsed with xylene. The stained sections were examined with transmitted light microscopy on a Nikon Eclipse 50i microscope (Nikon, Melville, N.Y.). All images were recorded with a DXM 1200F Nikon color digital camera.

Mechanical Characterization

To characterize the tensile properties of the neocartilage tissue in the 3D printed bionic ear, dogbone samples were 3D printed in the same chondrocytes density (~60 million cells/mL) as the bionic ear and at a lower density (20 million cells/mL) for comparison, and cultured under similar conditions for 10 weeks. Samples from various points in the culture were retrieved and uniaxial tensile testing was performed with an Instron 5848 Microtester (Instron, Canton, MA) [6] (Fig. S12). Prior to testing, the dimensions of the sample in the gauge area were measured using

a digital caliper. The samples were then clamped between serrated grips. A pre-load (< 0.5 N) was applied to ensure proper seating of the sample. The samples were then extended at a strain rate of 0.1% of their gauge length per second until failure occurred. Stiffness of the samples was determined from the linear region of the load-elongation curves. Young's moduli were calculated using the measured cross sectional area and gauge length.

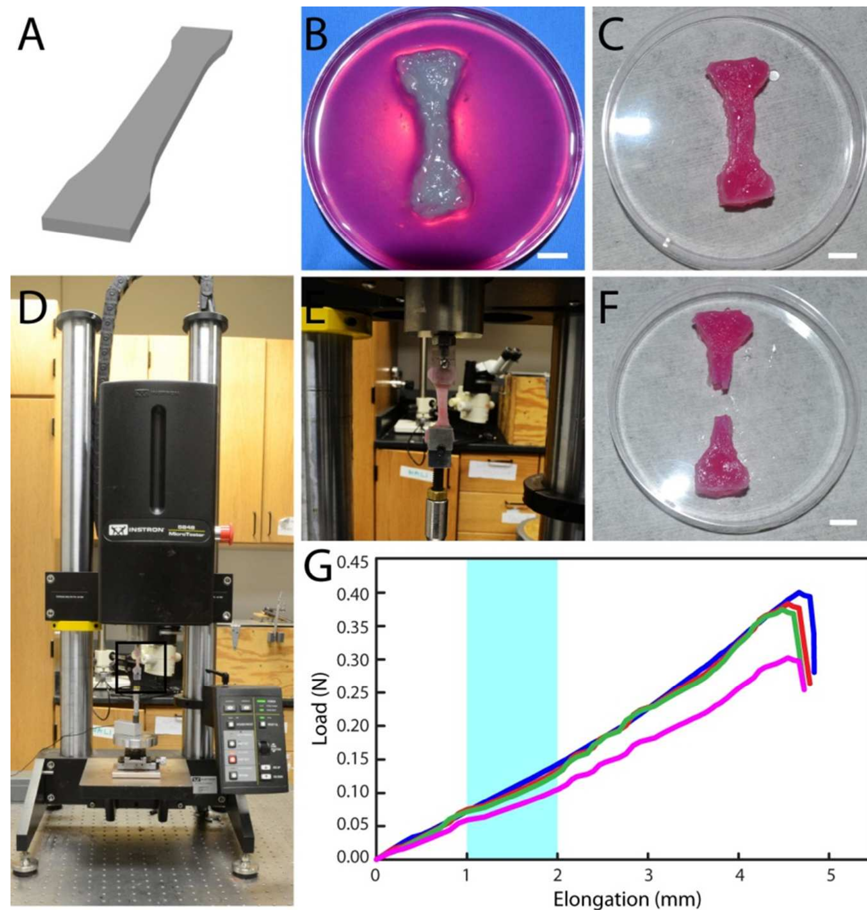

Figure S12. Tensile testing of 3D printed dog bone samples. (**A**) 3D model of the dog bone sample. (**B**) Image of the 3D printed dog bone sample under culture and (**C**) out of culture. (**D-E**) Tensile testing of the dog bone sample using Instron 5848 microtester. (**F**) Image of the sample after failure. (**G**) Representative load-elongation curves for the dog bone samples. Scale bars are 1 cm.

Hardness of the cartilage tissue after 10 weeks of culturing was determined by nanoindentation measurements at various anatomic sites of the ear auricle. Samples were tested on a Hysitron TriboIndenter (Hysitron Inc., Minneapolis, MN) using a 100 μm radius of curvature conospherical diamond probe tip[7] (Fig. S13). The round tip was chosen instead of a sharp tip to allow for better conformity during the contact to the tissue sample. A standard trapezoidal loading profile with a loading rate of 20 μN/s, a peak load of 200 μN, and a hold period of five seconds was applied in three repetitions to ten sites in each sample. The method of Oliver and Pharr was used to obtain reduced modulus (E_r) and hardness (H) from the unloading curves.[8] The reduced modulus is related to Young's modulus, E, by $1/E_r = (1-\nu_1^2)/E_1 + (1-\nu_2^2)/E_2$, where subscript 1 refers to the indenter material, subscript 2 refers to the indented material, and ν is Poisson's ratio. The ideal spherical tip function was used to calculate the projected contact area at the maximum load.

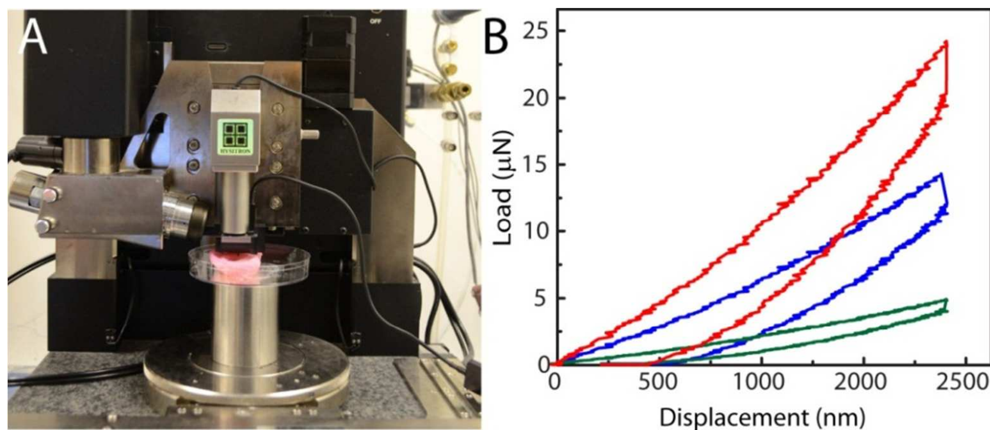

Figure S13. Hardness measurement of the 3D printed ear cartilage. (**A**) Image of the nanoindentation setup using a Hysitron TriboScope. (**B**) Representative load-displacement curves for the 3D printed cartilage.

Radio Frequency (RF) Hearing Experiments

Electrical measurements were performed on the bionic ear to demonstrate its ability to receive electromagnetic signals. First, self-inductances and intrinsic resistances of the bionic ear coil antenna and a wire-wound ferrite core inductor were measured using a Stanford Research Systems 785 dynamic signal analyzer. The bionic ear coil antenna of dimensions ~2 mm width and 1.8 mm thickness was found to have an intrinsic resistance of ~4 Ω up to point in the cochlear-like electrodes where electrical contacts are made. To demonstrate radio hearing of stereophonic sound, an audio signal containing left and right stereophonic music channels was sent to two wire-wound loop transmission antennas with ferrite cores. The left and right bionic ears were exposed to the signals transmitted by the corresponding antenna via magnetic coupling at a separation of approximately 0.5 cm. The audio signal picked up by the ears was then visually monitored in a digital oscilloscope by contacting the external cochlear-like electrodes of the ears. The received signal was also simultaneously played back in a loudspeaker.

Supporting Movies:

Movie 1. Video clip demonstrating the 3D printing of viable chondrocytes-seeded alginate hydrogel matrix in the precise anatomic geometry of a human ear auricle.

Movie 2. Video clip showing complementary left and right bionic ears demonstrating signal reception by listening to stereophonic audio music (Beethoven's "Für Elise").

References:

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