

Noninvasive Measurement of Ear Cartilage Elasticity on the Cellular Level: A New Method to Provide Biomechanical Information for Tissue Engineering

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Background: An important feature of auricular cartilage is its stiffness. To tissue engineer new cartilage, we need objective tools to provide us with the essential biomechanical information to mimic optimal conditions for chondrogenesis and extracellular matrix (ECM) development. In this study, we used an optomechanical sensor to investigate the elasticity of auricular cartilage ECM and tested whether sensitivity and measurement reproducibility of the sensor would be sufficient to accurately detect (subtle) differences in matrix compositions in healthy, diseased, or regenerated cartilage.

Methods: As a surrogate model to different cartilage ECM compositions, goat ears ($n = 9$) were subjected to different degradation processes to remove the matrix components elastin and glycosaminoglycans. Individual ear samples were cut and divided into 3 groups. Group 1 served as control and was measured within 2 hours after animal death and at 24 and 48 hours, and groups 2 and 3 were measured after 24- and 48-h hyaluronidase or elastase digestion. Per sample, 9 consecutive measurements were taken $\pm 300 \mu\text{m}$ apart.

Results: Good reproducibility was seen between consecutive measurements with an overall interclass correlation coefficient average of 0.9 (0.81–0.98). Although degradation led to variable results, overall, a significant difference was seen between treatment groups after 48 hours (control, 4.2 MPa [± 0.5] vs hyaluronidase, 2.0 MPa [± 0.3], and elastase, 3.0 MPa [± 0.4]; both $P < 0.001$).

Conclusions: The optomechanical sensor system we used provided a fast and reliable method to perform measurements of cartilage ECM in a reverse tissue-engineering model. In future applications, this method seems feasible for the monitoring of changes in stiffness during the development of tissue-engineered auricular cartilage. (*Plast Reconstr Surg Glob Open* 2017;5:e1147; doi: 10.1097/GOX.0000000000001147; Published online 9 February 2017.)

Reconstruction of the ear or nose after facial trauma such as severe burn injury or after tumor resection may be challenging especially when the cartilage

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framework has been damaged. Then, autologous cartilage grafts are conventionally used in combination with flaps such as the forehead flap for the nose or the temporo-parietal fascia flap for the ear. However, donor sites for cartilage grafts may be limited, and there is always a certain amount of donor-site morbidity.¹ In particular, reconstruction of cartilage structures such as the ear or alar nasi remains a challenge because their complex 3-dimensional shape and the high demands concerning the unique characteristics of cartilage.² The elastic properties of cartilage construct are relevant not only for clinical results but also for more fundamental research.

From the clinician's perspective, one tends to focus on the large-scale mechanics of transplanted tissues. To determine this, in general, gross mechanical testing is

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performed. Tensile properties of auricular cartilage and tissue-engineered cartilage, for example, were measured by Britt et al³ by applying an incremental tensile load to dumbbell-shaped cuts of cartilage. Another method was suggested by Roy et al⁴ who compared native, engineered auricular and costal cartilage in a 3-point bending apparatus mounted in a mechanical spectrometer. Compression loading experiments generally consist of either confined compression or indentation.^{5,6} Tensile or compression measurements all have their advantages and limitations.⁷ A crucial limitation they all share, however, is that the aforementioned methods only provide information on a gross anatomical scale. At present, many research groups are exploring the possibilities of regenerative medicine and tissue engineering of different facial tissues. Tissue-engineered ear cartilage for example could be an option to overcome tissue transplantation issues such as donor-site morbidity.⁸ The aforementioned mechanical testing is important from the surgical perspective as we need material of sufficient strength to maintain the shape when covered with muscle and skin for example. To design the appropriate scaffold for (cartilage) tissue regeneration, however, more detailed mechanical information on the extracellular matrix (ECM) level is crucial.

Essential for tissue engineering is creating the appropriate environment for the regenerative cells maintain their phenotype and form new tissue. The ECM is fundamental to the form and function of the tissue.⁹ Ear cartilage ECM consists of an intricate mix of collagen bundles, elastin fibers, and glycosaminoglycans each with specific effects on the mechanical properties of the tissue. Growing evidence suggests that cells are very susceptible to the mechanical properties of their environment.^{10,11} As such, the stiffness of the scaffold has an important influence on cell behavior and fate¹²⁻¹⁴ and thus on the mechanical traits of the construct as a whole. To form the correct tissue, it is essential that the scaffold closely matches the original stiffness of the damaged tissue. Specific spatial information on the biomechanics of the ECM at the microscale is, therefore, essential to clinical success; we need to be able to monitor the mechanical properties of tissue-engineered constructs on the cellular (micro) level over time. Regenerated cartilage will change mechanically as new ECM components and structures develop, and the scaffold degrades and becomes replaced. The quality assessment of tissue-engineered and transplanted constructs on the microscale, between atomic force microscopy (AFM) and gross mechanical testing, is therefore essential for future clinical implementation of regenerative medicine. To address this issue, we tested a commercial indentation device (Piuma Nanoindenter; Optics11, Amsterdam, The Netherlands) capable of measuring on the appropriate microscale. The system is based on a ferrule-top cantilever probe¹² whose utilization in the field of life science research has already been proven in different areas.^{13,14} Here, we extend this series of studies to assess its applicability to monitor ear cartilage regeneration over time using a reverse tissue-engineering model.

The response of cartilage to mechanical forces is influenced by 2 mechanisms: fluid dynamics and alterations

in the ECM structure itself.⁵ Glycosaminoglycans form an essential component of the ECM by attracting water and enabling osmotic pressure build-up.¹⁵ The distribution and mechanical function of elastin fibers are unique to ear cartilage.¹⁶ Their contribution to the specific mechanical properties of the ear makes it essential that at the time of implantation of the engineered cartilage, they have sufficiently formed. Conversely, removal of either of these components will qualitatively affect the cartilage. We designed an auricular cartilage tissue model to mimic the development of a scaffold over time in a reverse manner. Through specific enzymatic degradation of either of these components, we created an objective study model to test whether the indenter could accurately measure the differences between the various structural components of the ECM at the appropriate scale in a nondestructive matter. With the large attention and recent advancements such as 3-dimensional bioprinters for tissue regeneration, the ability to monitor the mechanical quality of developing tissues from beginning to end is crucial to the implementation of these novel tissue-engineering approaches in the clinic.

METHODS

Sample Preparation

Ear cartilage was obtained unilateral from 2- to 3-year old Dutch milk goats at a nearby abattoir. Ears ($n = 9$) were transported to the laboratory on ice where a $\pm 2\text{-cm}^2$ sized cartilage sample was taken from the base of each ear. The sample was further divided transversally into three 5-mm high sections and fixed on glass microscope slides with a thin layer of cyanoacrylate (super) glue. The transversal plane of each ear sample was measured fresh, and the same sample was then incubated in phosphate-buffered saline at 37°C for measurement at 24 and 48 hours. The other 2 samples were similarly incubated but with addition of a 1% solution of either hyaluronidase or elastase (both from Sigma Aldrich, Saint Louis, Mo.) to remove glycosaminoglycans or elastin, respectively.

Indentation Setup

Experiments were performed using a commercial nanoindenter (Piuma; Optics11). The instrument is capable of locally determining a sample's mechanical properties in both air and liquid environments by means of indentation. It utilizes ferrule-top cantilever probes^{8,22} to apply load and simultaneously measure the indentation depth using a fiber optic-based readout. The resulting stress-strain curves (Fig. 1A) are analyzed using the model described by Oliver and Pharr^{17,23} for a spherical indenter to determine the effective Young's modulus (E^*). The setup, using a suitable probe, is capable of applying forces ranging from 0.1 to 7.5 μN . Cantilever bending calibrations were performed before each series of experiments by indenting a rigid surface and equating cantilever bending to probe displacement. In many respects, this setup is comparable with an AFM. However, in contrast to most AFMs, which commonly function at subcellular (nano) ranges, this setup is also capable of functioning at cellular and tissue ranges.

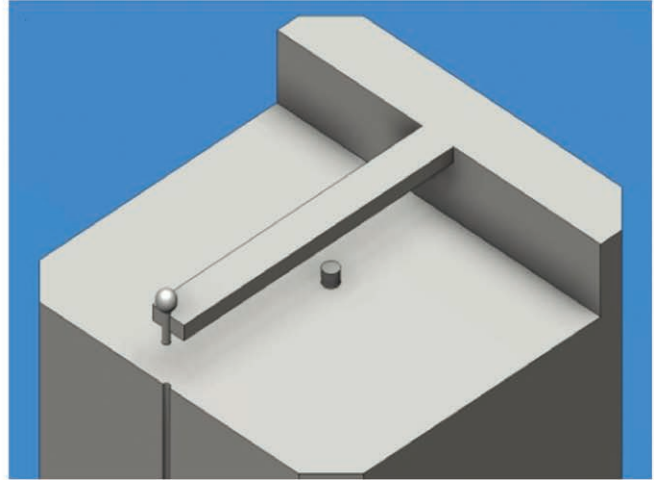
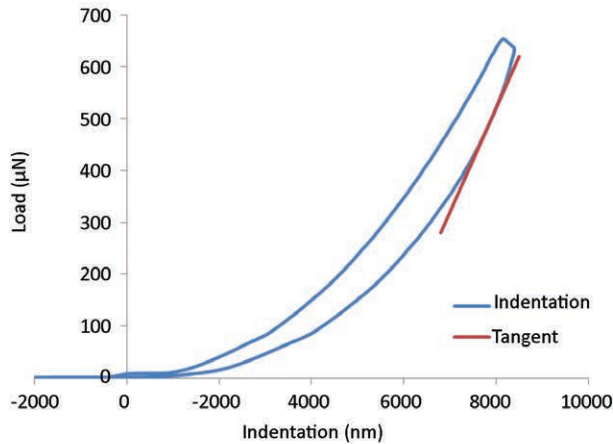


Fig. 1. A, Indentation curve of a cartilage measurement. Tangent (red) represents the slope of the unloading curve used to determine the effective Young's modulus. B, Graphic detail of the indenter tip: the ball forms the tip of the indenter ($\phi = 78 \mu\text{m}$).

Indentation Probe

A spherical indenter of $78 \mu\text{m}$ in diameter and a stiffness of 80 N/m was used to maximize the contact area and keep tissue damage during indentation to a minimum (Fig. 1B). To further increase the contact area with the tip, the indentation depth was maximized by using the full indentation depth of the setup ($20 \mu\text{m}$).

Indentation Protocol

The indentation protocol was carefully optimized to minimize viscoelastic effects from influencing the measurements. A 2-second loading period, 2-second hold period, and a 4-second unloading period were used, allowing accurate determination of curve angle coefficient.

Indentation Experiments

Samples were submerged in phosphate-buffered saline during experiments to prevent dehydration and minimize adhesive forces between the indenter and tissue surface. In preparatory experiments (data not shown), no detrimental effect of the glue on loading was found. Each sample was indented along the centerline of the cartilage to avoid edge effects. To average out possible surface roughness effects from the surgical cut and tissue inhomogeneity, each sample was indented on 9 locations $300 \mu\text{m}$ apart to obtain average data.

Histology

Three samples were fixated in 4% formalin and embedded in paraffin for sectioning according to standard protocols. Slides were stained with Alcian blue or Lawson stain to depict the glycosaminoglycans or elastin fibers respectively, and microscopic images were taken (Leica DM4000B/DC500; Wetzlar, Germany; magnification, $200\times$).

Statistical Analysis

Before the degradation experiments, the reproducibility of indentations was determined through the intraclass correlation coefficient (two-way random effect) of 10 con-

secutive measurements on a single location of 3 different ear cartilage samples. The variability between the separate indentations on the individual samples in the degradation experiment was calculated using the interclass correlation coefficient (ICC). Based on these results, the differences in effective Young's modulus were determined through univariate analysis of variance test. All analyses were performed using SPSS Statistics software version 22. A P value of less than 0.05 was considered significant.

RESULTS

An important aspect of these experiments was the definition of the indentation protocol to ensure that viscoelastic effects were neither contributing to nor influencing the unloading curve. Viscoelastic effects manifest themselves as a time-dependent dissipation of energy during indentation.¹⁸ This is visualized in the stress-strain curves by the area between the loading and unloading curves. The model described by Oliver and Pharr¹⁷ utilizes the slope of the unloading curve to determine the effective Young's modulus (Fig. 1A). To achieve this, a hold period was introduced after loading to give the samples sufficient time to allow energy to dissipate before unloading the sample. Next to this, the unloading speed was kept slow enough to allow the sample to readjust to the decreasing load as a build-up of delayed sample response would adversely affect the unloading curve. Preparatory experiments (data not shown) revealed that a 2-second load, 2-second hold, and a 4-second unloading period were sufficient in preventing viscoelastic effects from significantly influencing the unloading curve (linear drop in load values, thus allowing accurate determination of curve angle coefficient; Fig. 1A). Although the sample still displayed relaxation after the hold period in terms of indentation depth, a longer hold period did not indicate a significant change in energy dissipation during the complete indentation. The choice of the probe diameter was based on histological sections showing the average goat auricular chondrocyte diameter to measure around $10 \mu\text{m}$ and the

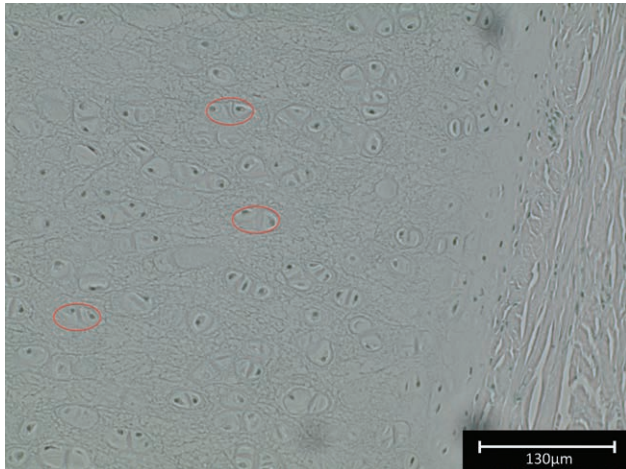


Fig. 2. Microscopic brightfield filter image (100x) of goat ear cartilage cross-section. Average lacuna size is approximately 40 μm (red ellipses).

more elliptical-shaped lacuna containing 2 or 3 chondrocytes at their longest measured around 40 μm (Fig. 2). As such, 78 μm was deemed sufficient to measure average ECM values while still being delicate enough to provide detailed information. Mechanical testing in the literature shows auricular cartilage to have a Young’s modulus ranging from 0.8 to 8 MPa.¹⁶ Using this as a reference, a cantilever with a stiffness of 80 N/m was selected to ensure that the majority of the probe’s stroke would be translated into indentation depth rather than cantilever bending, resulting in indentation depths between 7 and 15 μm.

The reproducibility of the indentation system was confirmed through repeated indentation at a single point on

3 different samples with an ICC of 0.99. Histology confirmed the degradation of glycosaminoglycan and elastin from the treated tissue samples in the peripheral zone where indentation experiments were performed (Fig. 3).

Good reproducibility was seen between the different measurements per individual sample with an ICC average of 0.9 (0.81–0.98). The native value for the ear cartilage effective Young’s modulus was found to be 5.2 MPa (±0.7). After hyaluronidase treatment for 24 and 48 hours, these values dropped to 2.2 MPa (±0.6) and 2.0 MPa (±0.3), respectively. Especially in the elastase group, individual differences varied considerably with an average effective Young’s modulus of 4.2 MPa (±0.62) at 24 hours and 3.0 MPa (±0.44) after 48 hours (Fig. 4). A slight decrease in the control group was also seen overtime probably due to autolytic processes, but a stable and significant difference ($P < 0.001$) was seen between all treatment groups after 48 hours (Fig. 5).

DISCUSSION

When clinicians think of tissue engineering, they often tend to focus only on the end results in terms of gross mechanical properties because from a clinical perspective, this aspect is vital to surgical success. However, it is now becoming more and more recognized that many tissue-engineered implants fail in the long term because of aberrant behavior of cells resulting in calcifications or deformation and resorption.¹⁹ Optimization of the biomechanical environment that cells will encounter may be pivotal for the steering of cellular differentiation and quality of matrix production. The ability to measure subtle differences in Young’s moduli of tissues and scaffolds is essential to properly fine-tune the cellular biomechanical micro-

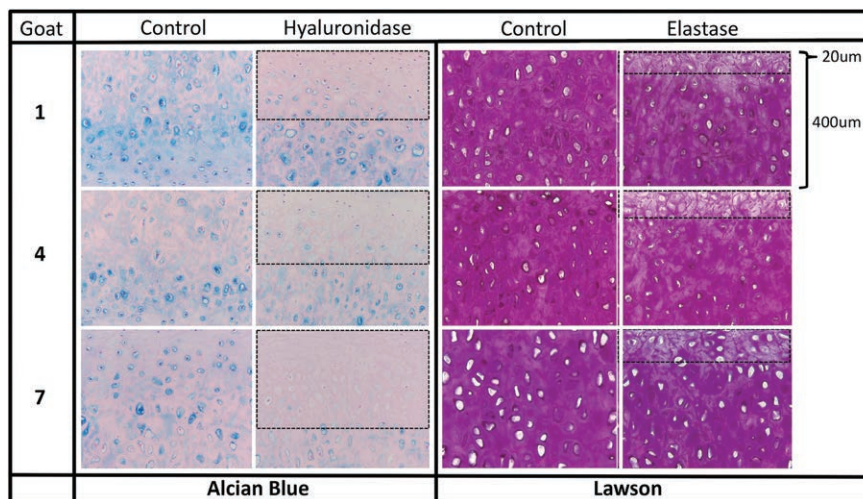


Fig. 3. Histological images (200x) of 3 random specimens (goats 1, 4, and 7) stained for glycosaminoglycan (Alcian blue stain, left section) and elastin (Lawson stain, right section) at 48 hours. All images were taken from the lateral sample side where indentations were performed (top of each image) and exposure to enzymes was maximal. Control samples of both groups show strong glycosaminoglycan or elastin staining. The samples treated with hyaluronidase or elastase show reduced stain intensity, especially in the lateral (indented) areas as depicted by the dotted boxes. Enzyme penetration and subsequent degradation were more than sufficient and far exceeded the maximum indentation measurement depth (>20 μm).

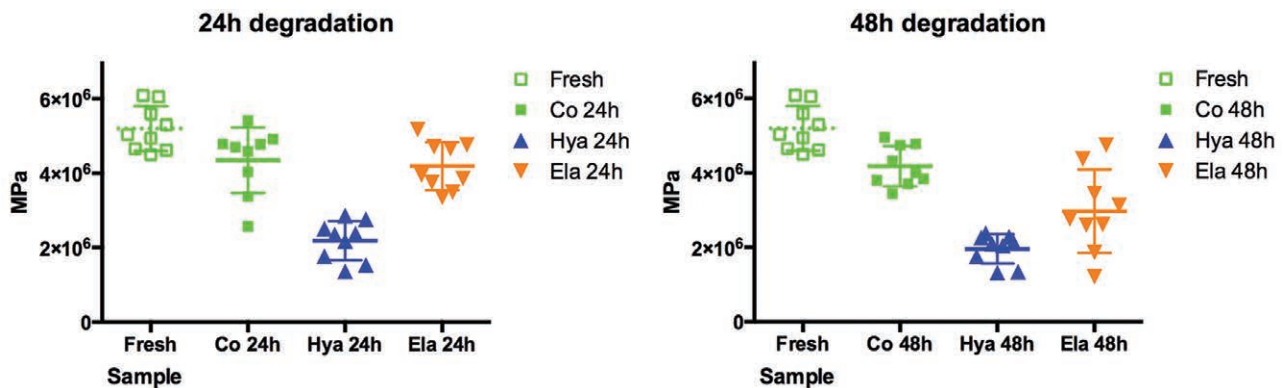


Fig. 4. Young's modulus of different samples per group at 24 hours (left) and 48 hours (right). Co, control; Ela, elastase-treated group; Hya, hyaluronidase-treated group.

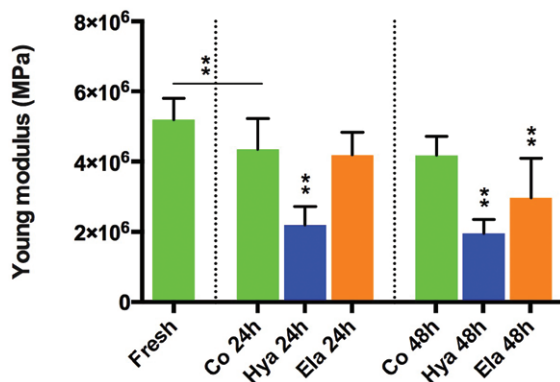


Fig. 5. Average Young's modulus of sample groups after 24- and 48-h degradations (** $P < 0.001$). Control groups showed an initial loss of stiffness after 24 hours but no further decrease at 48 hours. The elastase-treated group (Ela) showed no significant loss of stiffness after 24-h enzyme exposure unlike the hyaluronidase-treated group (Hya). At 48 hours, all treated groups showed significant reduced stiffness compared with the control group.

environment. In this study, we investigated a new tool capable of measuring tissue stiffness on the cellular level, which allows nondestructive assessment of not only the final characteristics but also the starting conditions and in-process quality of developing tissues and constructs, thereby boosting the development of novel, efficacious tissue-engineering approaches.

We chose the “reverse tissue-engineering model” using goat ear cartilage as a consistent source of fresh material to demonstrate the applicability of this new technology to measure tissue elasticity in a variety of compositions. To explore the applicability and sensitivity of our setup to detect subtle differences in ECM quality and composition during the tissue (re)generation process using tissue stiffness as the outcome parameter, we treated mature elastic cartilage with different degradation enzymes. The removal of either elastin or glycosaminoglycan resulted in significant general decrease of elasticity. The large differences in the elastase digestion group, however, were surprising. Smith et al²⁰ already noted that their extensive protocol for enzymatic degradation of elastic fibers in the annulus fibrosus of intervertebral discs was not absolute. Especially

in the elastase group, they reported high variability. They further suggested that degradation of elastic fibers results in separation and deformation of the collagen structure. This could explain why we found such a large variety in our elastase group; in other words, it may be because of varying elastin concentrations and subsequent degradation between animals in combination with rearrangement of the remaining collagen matrix. The latter could also explain the initial slight increase in elasticity of some samples. This effect had also been observed in a study on the effect of elastase on porcine carotic artery wall.²¹ Elastase degradation was associated with enlargement of the arterial wall but not with stiffening or softening. The structural change of ECM subsequent to the alteration of elastin fibers has also been suggested as the reason why ears increase in size with old age.²²

In summary, it is crucial to determine the mechanical properties on a (sub)cellular level to gain insight in the optimization of extracellular mechanics. The optomechanical sensor system we used provided a fast and reliable method to perform measurements of cartilage ECM changes on this level. Currently, we are exploring the possibility to combine the indentation system with optical coherence tomography, which will allow concomitant visualization of the tissue architecture while indenting. As such, it will be possible to discern the stiffness of different tissue layers in a noninvasive fashion. In the future, this may provide us with an essential clinical tool to determine the quality of transplanted or regenerated tissue in facial reconstruction.

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