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HIGH FREQUENCY ULTRASONIC IMAGING OF GROWTH AND DEVELOPMENT IN MANUFACTURED ENGINEERED ORAL MUCOSAL TISSUE SURFACES

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Abstract

This study uses high-resolution ultrasound to examine growth and development of engineered oral mucosal tissues manufactured under aseptic conditions. The specimens are a commercially available natural tissue scaffold, AlloDerm[®] and oral keratinocytes seeded onto AlloDerm[®] to form a *ex vivo* produced oral mucosal equivalent (EVPOME) suitable for intraoral grafting. The seeded cells produce a keratinized protective upper layer which smooth's out any remaining surface irregularities on the underlying AlloDerm[®]. Two-dimensional acoustic imaging of unseeded AlloDerm[®] and developing EVPOMEs were performed at each consecutive day of their growth and development, requiring imaging each of the tissue specimens under aseptic conditions (total time from seeding to maturation: 11 days). Ultrasonic monitoring offers us an ability to determine the constituents in the EVPOME that are responsible for changes in its mechanical behavior during the manufacturing process. Ultrasonic monitoring affords us an opportunity to non-invasively assess, in real time, tissue engineered constructs prior to release for use in patient care.

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Keywords

Acoustic elasticity microscopy; oral mucosa; non-invasive assessment (tissue engineering, ultrasound); image analysis; keratinocyte; cell spreading

INTRODUCTION

Scanning acoustic microscopy (SAM) has been shown to be an effective tool to study both the mechanical properties and non-linear elastic characteristics of cells and tissues [Cohn et. al. 1997a; Cohn et. al. 1997b; Hollman et. al. 2002; Kolios et. al. 2002]. The advantages of using SAM over conventional light and electron microscopy include being able to image the cells and tissues without damaging the cells or alter the tissues; this provides a more accurate representation of the tissues' natural properties [Cohn et. al. 1997a]. We have compared SAM imaging results to standard optical microscopy [Winterroth et. al. 2011a], followed by single-blind studies comparing engineered tissue specimens to those subjected to an elevated thermal stress (damaging the cells) [Winterroth et. al. 2011b]. We further tested the physical elastic properties of the *ex vivo* produced oral mucosal equivalent (EVPOME) and the commercially available unseeded acellular cadaveric dermis, AlloDerm® (LifeCell Corp., Branchburg, NJ, USA) which serves as the scaffold for the EVPOME construction and development [Wagner et. al. 2009].

Previously, we used SAM to compare any changes in the radiofrequency (RF) data to the EVPOME and natural human oral mucosal cells undergoing differentiation, apoptosis, and keratinization [Winterroth et. al. 2009; Zuber et. al. 1999]. The spectral analysis results from SAM can be compared to histological images of the EVPOME tissues at different stages of growth and development. By correlating changes in the RF data to the EVPOME (and mucosal cells in general) undergoing differentiation, apoptosis, and keratinization, we can better understand the physiological processes of these cells as they evolve and proliferate along the AlloDerm® surface.

AlloDerm® is demonstrated as a viable scaffold for producing the engineered oral mucosal tissue. It is obtained from allograft donor skin and produced by a carefully controlled process that removes the epidermis and dermis cells without altering the extracellular matrix structure while maintaining an intact basement membrane, thus reducing the immune responses and the transmission of diseases [Harrison et. al. 2006; Vendramini et. al. 2006; Wagner et. al. 2009].

SAM is an effective tool to study both the morphology and non-linear elastic characteristics of natural and engineered oral mucosal tissues [Cohn et. al. 1997a]. Non-invasive and non-destructive imaging of cells and tissues not only gives accurate assessments of these organisms (as they are alive when being imaged), it also provides evidence as to the degree of differentiation which the cells are undergoing *in situ* [Holland et. al. 1997; Kolios et. al. 2003; Saijo et. al. 2004]. Examining the acoustic properties of tissues also allows us to study its density and elasticity.

The reflectivity off the superior portion of the EVPOME allowed us to quantify the degree of surface roughness, which showed a strong linear correlation in quantification of the surface characteristics between the optical and SAM imaging [Winterroth et. al 2011a].

Although SAM was used to study the morphology and density of skin tissue under both normal and pathological conditions [Barr et. al. 1991], it has not been applied toward understanding growth and development of the cellular component and finalized engineered tissues during its manufacturing process. as was done in a recent study involving Raman spectroscopy (Khmaladze et. al. 2012). In this study we successfully utilized the SAM to assess the cellular component and final tissue construct of the EVPOME during its manufacturing process.

MATERIALS AND METHODS

Tissue Preparation – EVPOME

Methods for preparing both AlloDerm® and EVPOME devices are similar to those described elsewhere [Izumi et. al. 2004]. Briefly, oral mucosa keratinocytes were enzymatically dissociated from the tissue sample, and a primary cell culture was established and propagated in a chemically-defined, serum- and xenogenic products-free culture medium, with a calcium concentration of 0.06 mM. The AlloDerm® specimens were soaked in 5 µg/ml of human type IV collagen overnight at 4°C prior to seeding cells to assist the adherence of cells, then approximately 2.0×10^5 cells/cm² of oral keratinocytes were seeded onto the type IV collagen pre-soaked AlloDerm® and cultured in medium with 1.2mM calcium. The composites of keratinocytes and AlloDerm® were then cultured, in the submerged condition, for 4 days to form a continuous epithelial monolayer. At Day 4 of cell post-seeding, samples of the EVPOME were collected while in the submerged condition for SAM imaging. After 4 days, the equivalents were raised to an air-liquid interface with an increase in calcium concentration to 1.8mM to encourage epithelial stratification and cultured for another 10 days, resulting in a fully-differentiated, well-stratified epithelial layer on the AlloDerm®. AlloDerm® specimens (used as controls) were treated in the same manner as EVPOMES with the exception that they were never seeded with cells, oral keratinocytes. The set up for all of the tissue specimens when scanned is shown in Figure 1. Each specimen was approximately 1.0 cm² in area.

A total of three scanning sessions were conducted for this study, with three specimens prepared - one AlloDerm® and two EVPOMES - for each session. We started the SAM scans of the specimens at Day 4 post-seeding.

SAM Logistics: Set-Up and Imaging

Details for the set-up of the in-house built SAM have been detailed previously [Cohn et. al. 1997a; Cohn et. al. 1997b; Hollman et. al. 2002]. Briefly, AlloDerm®, EVPOME, and natural mucosal tissue samples were immersed in deionized water and imaged with a single element fixed focus transducer (NIH Resource Center for Medical Ultrasonic Transducer Technology at the University of Southern California, Los Angeles, CA., USA) producing ultrasonic B-scans. The transducer has an approximate frequency of 50 MHz; the element is

3 mm in diameter and focused to a depth of 4.1 mm, giving an f-number of approximately 1.4. The transducer was fastened to an optical mount and the angular position was adjusted until the ultrasonic beam was normal to the deflecting plate. Stepper motors control the positioning motion of the transducer; the stepper motors are controlled by a stepper motor driver (MID7604, National Instruments, Austin, TX. USA) and motion control card (PCI-7354, National Instruments, Austin, TX. USA) installed in a desktop computer. In the SAM 2-D B-scan for the specimens, the transducer is positioned at the top of the image, pointing downward. The transducer's parameters are: 15 μm scanning step size in both the transverse and horizontal directions; a lateral resolution (R_{lat}) of 37 μm ; an axial resolution (R_{ax}) of 24 μm ; a depth of field of 223 μm ; the f-number is 1.5. Z-axis was sampled at 300 mega samples/second. Axial resolution is the resolution in the direction of propagation and is determined by the length of the ultrasound pulse propagating in the tissue; lateral resolution is the resolution orthogonal to the propagation direction of the ultrasound wave. Table 1 provides additional details on the SAM's operation.

We scanned the surfaces of the EVPOME and AlloDerm[®], showing the acoustic signal between the interface of the sample and water on the sample's apical side. DC stepper motors accurately positioned the transducer above the specimen. B-scan images were obtained by stepping the transducer element laterally across the desired region. At each position, the transducer fired and an RF A-line was recorded. After repeated firings at one position, the transducer was moved to the next position, where the image was constructed from A-lines acquired at all lateral positions. Because of a low ratio of aperture size (namely the transducer's diameter) to imaging depth (f-number), single element transducers have a short depth of field; a composite B-scan image was generated from multiple scans at different heights.

The top bright echo indicates the boundary between the coupling medium (water) and the apical surface of each specimen. Below this, the specimen appears as uniform speckle; there is no second boundary between the tissue interface and the lower tissues. True surfaces from the specular reflections are rendered at the threshold value, not at the peak. The large bright spots indicate backscatter and are approximately 30 μm in diameter. Based on this reflectivity (namely, the degree of the ultrasonic signal that it reflected back to the transducer; the greater the reflected signal from any given material will produce a brighter image in the B-scan), there is a much brighter, more uniform surface layer in the SAM EVPOME images as they grow and mature; in contrast, the images of the unseeded AlloDerm do not display this bright surface layer. SAM images are derived from the differences in the acoustic patterns as they reflect off of the tissue boundaries (surface and base) [Saijo et. al. 1991; Hollman et. al. 2002], including the phase shift in the sound waves when reflecting off the tissue as opposed to the base surface of the holder, the reflections off the surface and bottom of the tissue, and the sound speed through water and tissue. The tissue surface was determined by thresholding the magnitude of the signal at the first axial incidence of a value safely above noise, approximately 20–30 dB (Figure 2). All of the scans took place under aseptic conditions within a laminar flow hood. Each specimen was individually scanned in a sterile petri dish containing sterile culture media.

Between 2–4 specimens (both AlloDerm[®] and EVPOMEs) were scanned for each experiment set. Each specimen was scanned twice in randomly selected areas of their surfaces for every consecutive SAM scanning day (Days 4 – 8 and Day 11 post-seeding) in each study throughout its manufacturing process. Each specimen was returned to an incubator kept at physiological temperature and CO₂ (37°C; 5% CO₂) after completing its scanning session.

Surface profilometry was determined by first finding the instance of threshold value, fitting and subtracting the planar surface, then calculating root-mean-squared (RMS) height. RMS was computed in time domain:

$$\text{RMS} = \sqrt{\frac{1}{n} \sum_n x^2(t)} \quad (1)$$

where n is number of $x^2(t)$ samples.

Histology

AlloDerm[®] and EVPOME (at different stages of growth following seeding of cells) were fixed with 10% formalin, embedded in paraffin, cut in 5 µm sections, and stained with hematoxylin and eosin. The specimens were then examined under a Nikon Ti-U inverted brightfield microscope (Nikon Optical, Tokyo, Japan).

Statistical Analyses

The RMS values for the individual specimens. AlloDerm[®] and EVPOME, were calculated in order to quantify the surface irregularities on both specimens imaged using both SAM and histological preparations. We fit the planar surface for all specimens and determined the number of variations in the tissues above and below the surface. A known area from each micrograph examined (300–1000µm × 100µm) was divided into 12–40 even segments: 4 rows, 4–10 columns; the total area for each divided segment was 2610µm². Each segment was then examined for the number of irregularities between the foreground (tissue and device) and background (empty slide). The number of times the foreground (device) and background (empty slide) appears in each segment was then quantified for each row. The numbers for all rows were then summed, giving the total counts for the entire area; the number of pixels of the surface differences between the unseeded AlloDerm[®] scaffold and the EVPOME at each successive day post-seeding were counted. Counts were taken every 100 µm along 1000 µm of each specimen's surface and the mean numbers for each specimen, AlloDerm[®] and EVPOME, were calculated. Figure 3 provides an illustrative example of how the counts were obtained.

We performed ANOVA on the surface characteristics similar to our earlier studies (Winterroth 2011a) - data is not shown). P-values were calculated based on differences in the number of surface irregularities between the developing EVPOME and the unseeded AlloDerm[®] (data not included). Based on the values being extreme, we rejected the null hypothesis as there is a relationship between the growth/development of the EVPOMEs and the surface characteristics.

RESULTS

None of the samples showed visible signs of contamination at the experiment's conclusion. SAM 2D B-scan images of the AlloDerm® and EVPOME (Days 4 – 8 and 11 post-seeding) are shown in Figure 4, along with their histology counterparts in Figure 5: images in both figure sets show clear differences in the surface between the unseeded AlloDerm® control and increased EVPOME maturation at each successive day. In the EVPOMEs, the surfaces show the gradual formation of the space-filling keratinocytes and their differentiation between Days 5 – 7 (Figures 4 – 5). The SAM B-Scans show greater reflectivity at each successive scan day, evident by the appearance of the bright white band at the top of the specimen; this band noticeably increases in size and intensity at each progressive day of the scan sessions. This is indicative of the space-filling/keratinization production by the cells on the EVPOME surface and appears to maximize between Days 7 – 8, becoming fully mature at Day 11; the histology images again validate these findings (Figure 5).

Further, quantifying the surface differences between the AlloDerm® and EVPOME at each successive day of development showed decreases in the surface inconsistencies of the EVPOME. As the EVPOMEs developed, there were fewer changes on their surfaces (Figure 6); this correlates with the surface reflectivity of the B-Scans and in the keratin and/or stratification visible in the histology images. No significant differences were observed for the AlloDerm®, either at the start (Day 4) or end (Day 11) for each scan session (Figure 6); because they are unseeded, no growth or space-filling is expected, hence differences are negligible.

The RMS values between the unseeded AlloDerm® and EVPOME's showed little to no significant differences between the two specimens throughout the course of the EVPOMEs' manufacturing process. However, comparing the standard deviation between the two specimens showed significant variance between the two: the AlloDerm® clearly showed greater deviation than the EVPOME (Figure 7). Such deviation was apparent for each consecutive scan day throughout the duration of each scan session.

DISCUSSION

We demonstrated SAM's efficacy to non-invasively and aseptically monitor growth and maturation of the cells and tissue, during the manufacturing process.

There were several problems encountered when performing the aseptic scans using our aforementioned methods. The variability seen in the RMS profiles and B-Scans are the result of high noise levels above the threshold values. Unlike our previous scans, the specimens could not be secured to the base of the petri dish (Figure 1), possibly adding to the noise above the surface of the tissue. We further encountered difficulties when attempting to aseptically scan the tissues directly out of the petri dishes: because of the shallow depth of the dishes, achieving a proper focal length was difficult. If we retracted the transducer too far from the specimen, it would occasionally break the surface tension of the fluid, causing scatter and hence, ruin the scans. Merely adding fluid into the petri dish did not always correct this problem as the specimen then tended to shift. We subsequently

switched to using a 16-well (each of the wells being approximately 2.0 cm in diameter) plate which corrected these problems

The SAM scans did not show significant RMS differences between the unseeded AlloDerm® and mature EVPOME except in the start and conclusion of the manufacturing process (Figure 7). Nevertheless, the significant difference in the standard deviation even at the early stages of the EVPOME growth demonstrates two major findings: We can quantitatively differentiate any variation between the two specimens, and the SAM can visually display and quantitatively assess the growth and differentiation of the EVPOME throughout its manufacturing process (Figures 4 and 7).

Repeating the SAM scans for each of the EVPOME specimens at each consecutive day showed the expected decreases in surface irregularities and increased keratinization as the EVPOME developed and matured; details of this surface maturation were confirmed by our earlier study [Winterroth et. al. 2011a]. The high RMS variance is likely due to the greater numbers of surface irregularities present on the AlloDerm®; these are also noticeable on the EVPOME during the first 3–4 days after seeding them with cells. For the EVPOME, these surface irregularities decrease as the cells proliferate and fill in the surface irregularities, followed by producing keratin on the surface. The SAM scanning and RMS values we quantified for the keratinocyte maturation can potentially be applied to other cells and tissue cultures. The non-invasive properties of SAM make it a highly accurate and effective means for imaging live biological specimens.

The particular decrease in the surface irregularities occurs between Days 5 and 6 post-seeding; the most active time of development, at least for the keratinization of the surface. The B-Scans on the EVPOME shows a distinct development on the surface through those active days of maturation: increasing keratinization and space filling at each successive day of growth (Figures 4 and 5). This necessitates greater surface profilometry studies to analyze its specific growth and development characteristics.

Earlier, we profiled the nonlinear stress-strain characteristics between unseeded AlloDerm® and mature EVPOME showing clear, significant differences between the two. Results showed a near 2.5-fold difference in the stiffness properties between the manufactured EVPOME and the unseeded AlloDerm® [Winterroth et. al. 2012].

Our future studies mandate studying exactly what specific cellular/tissue constituents contribute to these aforementioned stress-strain differences. Further, we must examine exactly at what stage(s) in the maturation of EVPOME do these differences occur and which components (cells, keratin, or a combination) are responsible for them.

We have shown how SAM is a non-invasive *in vitro* quality control method for imaging engineered oral mucosal tissues, cornea, and potentially other soft tissues [Hollman et. al. 2002; Winterroth et. al. 2011a]. Our previous studies on thermally stressed EVPOME with SAM showed that it was able to differentiate between an good versus failed EVPOME [Winterroth et. al. 2011b]. This current study also serves as a segue for us to examine at exactly what time point(s) EVPOME begins to fail in addition to exploring other time course studies using SAM.

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Figure 1. Basic SAM set-up of the AlloDerm® and EVPOME (at different days post-seeding) for imaging. The transducer was immersed into the culture medium and imaged under sterile conditions each day for the duration of the EVPOME's growth and development.

Figure 2.

Plot diagram of a typical SAM scan (of what?). This shows the detection of the specimen's surface (red line) after setting the threshold detection safely above the signal of the water.

Figure 3.

Illustrative set-up exemplifying how the surface irregularities were counted for both the AlloDerm® and EVPOME images. A known area from each micrograph examined ($300\text{--}1000\mu\text{m} \times 100\mu\text{m}$) was divided into 12–40 even segments: 4 rows, 4–10 columns; the total area for each divided segment was $2610\mu\text{m}^2$. Each segment was then examined for the number of irregularities between the foreground (tissue and device) and background (empty slide). The number of times the foreground (device) and background (empty slide) appears in each segment was then quantified for each row. The numbers for all rows were then summed, giving the total counts for the entire area; the number of pixels of the surface differences between the unseeded AlloDerm® scaffold and the EVPOME at each successive day post-seeding were counted. Illustration is not to scale.

Figure 4.

B-Scans of the EVPOME development between Days 4 and 11 post-seeding. The bright white bands show space-filling and keratinization over the surface irregularities in images (arrows), increasing as the EVPOME develops and matures. Any remaining surface irregularities are absent by Days 7 through 11. Unseeded AlloDerm® images are visibly unchanged at Days 4 and 11. Scale bars equal 100 μm .

Unseeded AlloDerm® - Day 4 **EVPOME - Day 4** **EVPOME - Day 5** **EVPOME - Day 6** **EVPOME - Day 7** **EVPOME Day 8** **Unseeded AlloDerm® - Day 11** **EVPOME – Day 11** **Figure 5.**

Histology images of the unseeded AlloDerm® and EVPOME during its development between Days 4 and 11 post-seeding. There are areas showing keratinization of surface irregularities in images (arrows), increasing as the EVPOME develops. Any remaining surface irregularities are virtually absent and fully keratinized by Days 7 through 11 (asterisks). Unseeded AlloDerm® images are shown unchanged at Days 4 and 11. Scale bars equal 100 μ m for the AlloDerm® image (at Day 4 only) and 50 μ m for each EVPOME image and the AlloDerm® image at Day 11.

Figure 6.

Mean pixel counts of the surface differences between the unseeded AlloDerm® scaffold and the EVPOME at each successive day post-seeding. Counts were taken every 100 μm along 1000 μm of each specimen's surface.

Figure 7.

Comparing the RMS values from the SAM images between the unseeded AlloDerm® scaffold and the developing EVPOME between days 4–11 post-seeding. Although there is little change in either specimen's values (both specimens remain between 14–17 μm throughout the duration of the experiments), there is a significantly greater difference in the standard deviation among the AlloDerm® compared to the EVPOME.

Table 1

Basic equations of the SAM operating principles: axial and lateral resolutions, DOF, and acoustic impedance.

The axial resolution depends on the dimensions of the pressure pulse, which is related to the transducer bandwidth (BW) and system electronics:

$$R_{ax} = \frac{c}{2 \times BW} \quad (1)$$

where c is the sound speed in the tissue and BW is 32MHz. Likewise, the lateral resolution at the focal point can be estimated by:

$$R_{lat} = \lambda \times f / \text{number} \quad (2)$$

where, λ is the wavelength (25 μ m). The depth of field (DOF) is calculated by:

$$DOF = 4 (f / \text{number})^2 \lambda \quad (3)$$

Due to a small f/number (which provides a tight beamwidth), the DOF is also limited. Lateral resolution is the resolution orthogonal to the propagation direction of the ultrasound wave. Acoustic Impedance (Z): The impedance determines the amplitude of the reflected / transmitted waves at the fluid-tissue interface. Complex scattering properties of tissues are due to acoustic impedance interfaces in microstructure of tissues:

$$Z = \rho c \quad (4)$$

$$R = |(Z2 - Z1) / (Z2 + Z1)| \quad (5)$$

Where Z is the impedance, ρ is the density, and the reflection coefficient (R) is the ratio of the impedance mismatch ($Z1, Z2$) between the two materials – fluid and tissue.