Engineering Skin and Measuring Skin Toxicity

Julianne Jorgensen, Michael Sheets, Morgan Zhu
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I. Introduction

With an increase in (1) transdermally applied drug treatments (patches and gels), (2) alternatives to animal tested make-up, and (3) recognition of percutaneous absorption of organic solvents as occupational health hazards, accurate bioengineered *in vitro* transdermal models for drug testing are needed to ethically study and quantify the effects of long-term exposure to these agents. As an engineered tissue, skin has received much attention for its potential as a useful transdermal drug delivery model [1,2]. Currently, the most accurate model possible is human cadaver skin or neonatal foreskin samples [1]. However, as these skin models are difficult to obtain and maintain after harvest, excised animal (usually murine) or engineered models (epidermal and full skin models) are often used, each with their limitations [2].

With the limited availability of cadaver tissue, excised animal tissues are commonly used as a model system for chemical absorption through skin. However, it has been found that the structure of skin from common laboratory animals (rabbit, rat, mouse, guinea pig) results in consistently higher permeability, increased cellular uptake, and higher levels of toxicity compared to human skin [1,3,4,5]. While some species (pig and monkey) may offer a more adequate approximation for human skin, variations in sources for the samples make results difficult to compare across publications [3].

These shortcomings have been addressed by using engineered tissue with human cells. Nonetheless, various engineered skin models each have their own shortcomings. Simpler epidermal models may be a poor predictor of toxicity due to their lack of a dermal layer [1]. More advanced human skin models with multiple cell types often require neonatal foreskin, tissue samples obtained during cosmetic surgery, amputations, or excised cadaver tissue to provide initial cells for the engineered construct [1, 4]. These models also require keratinocytes, which are difficult to maintain after harvest [2].

One way around the issues associated with engineered skin using human cells is to use bone-marrow derived mesenchymal stem cells (BMSCs) due to their proliferative and differentiation capacities [2]. Ma *et al.* demonstrated that BMSCs can behave as fibroblast-like cells in the dermal layer and differentiate into keratinocyte-like cells in the epidermal layer when co-cultured in a collagen matrix in the presence of an air-liquid interface [2]. The resulting epidermal layer was shown to express cytokeratin, a marker specific to epithelial cells, and mimic the stratification patterns associated with keratinocyte cultures [2]. However, the mechanical and functional (barrier-like) properties of this construct were not investigated.

This paper seeks to explore the efficacy of BMSC skin constructs in a bovine model. An exploratory phototoxicity study was performed using neutral red and sodium dodecyl sulfate (SDS) [6]. Phototoxicity is an interesting application of skin models, as radiation from the sun can change the effects of a drug, and early testing of this data without the need for animal or cadaver models could be useful for transdermal drug testing [7].

II. Materials and Methods

A. Reagents

Dulbecco's modified Eagle's medium (DMEM, GE Healthcare), Ham's F12 nutrient mix (Lonza), penicillin-streptomycin solution (Lonza), and 40,6-diamidino-2-phenylindole, dihydrochloride (DAPI, Thermo Scientific) were purchased from Fisher Scientific Corporation. Hydrocortisone, insulin, neutral red, and secondary FITC-conjugated goat anti-murine Ab were purchased from Sigma-Aldrich. 1α,25-Dihydroxyvitamin D3 (VD3) and 3,3′,5-Triiodo-L-thyronine (T3) were purchased from Caymen Chemical. Type I rat tail collagen was obtained from Becton Dickenson. Epidermal growth factor (EGF) was purchased from ProSpec. Monoclonal murine anti-pan keratin was purchased from Biolegend Inc.

B. Cell isolation and compaction study

Primary bovine bone marrow-derived mesenchymal stem cells were harvested from calf knees as previously described [8]. Cells were expanded to 70-80% confluence in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum, 100 IU/ml penicillin and 100 µg/ml streptomycin, 1 ng/mL basic fibroblast growth factor (bFGF), and 0.1 mM nonessential amino acids (expansion media).

Constructs were prepared as previously described [2]. Type I rat tail collagen was diluted to a final concentration of 1.5 mg/mL in sterile PBS with phenol red and titrated to pH 7.4 with 1M NaOH. In order to determine optimal compaction conditions, 300 µl of the collagen mixture was added to each insert in a 12 mm Corning Transwell plate and allowed to gel for 30 minutes at 37°C. BMSCs (passage 3-5) were then

added to the top of the acellular collagen mixture at a density of 125,000, 250,000, or 500,000 cells per well and allowed to gel. Compaction was measured over six days by removing each insert and measuring the height of the gel using digital calipers. Calipers were sterilized, held up next to the insert, and adjusted to visually line up with the collagen height. The minimal cellular density required to achieve 40% compaction after six days (typical compaction levels for fibroblast-seeded dermal constructs [9]) was chosen as the optimal seeding density.

C. BMSC differentiation time course

BMSCs were cultured in a 3:1 mixture of DMEM and Ham's F12 nutrient mix containing 10% FBS, 100 IU/ml penicillin and 100 µg/ml streptomycin (Fisher Scientific), and HITED differentiation media [Ma *et al* 2009] on coverslips and fixed in 4% paraformaldehyde (PFA) at either 1, 4, 7, or 10 days. Expression of pan-keratin was characterized via immunostaining with purified monoclonal murine anti-cytokeratin (pan-reactive) and followed by FITC-labeled goat-anti-murine at each time point.

D. Preparation of full skin constructs

Dermal gels (acellular and cellular layers) were prepared as described above with the chosen seeding density and allowed to compact for seven days. 500,000 BMSCs per well were then seeded on top of the collagen matrix and fed with 0.5 mL HITED differentiation media from below the construct. The construct was then cultured for 14 days and fed daily with HITED media from the bottom chamber, as shown in Fig. 1. Each of the three cell concentrations were made and measured in triplicate.

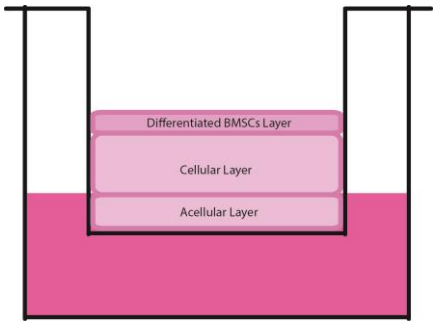

Fig.. 1: Layout of engineered skin constructs. Construct was formed from a bottom acellular collagen layer, a cellularized BMSC collagen layer, and surface seeded with BMSCs to encourage keratinocyte differentiation due to the air-liquid interface. Constructs were bottom fed with HITED differentiation media.

E. Drug & Phototoxicity Testing

Media was aspirated from constructs, and a circular piece of filter paper was placed on each construct (except on 'No Filter' controls). 200ul of either 0.01% neutral red, 0.05% SDS, or tissue-culture grade water was placed on each pad of filter paper, and cells were fed with DMEM. Cells were incubated at 37°C, 5% CO_2 for 24 hours [10].UV-treated samples were irradiated with with a UVP UVGL-58 handheld lamp at 254 nm for 30 minutes. All constructs were washed with PBS and incubated for a 24 hour recovery period in DMEM before fixing in 4% PFA.

F. Histology & Staining

Samples were fixed in 4% PFA at 20°C for 24 hours, embedded in paraffin, and sectioned using a rotating microtome. Immunohistochemistry was performed using murine anti-pan-keratin and FITC-labeled goat anti-murine and counterstained with DAPI.

G. Mechanical Testing

1) Hardness

Sample hardness was evaluated with a shore 00 hardness tester. The tester was held at a 90^0 angle above the sample and gently pressed until flush with the sample. Three replicates were used for each time point. Samples were tested three times each at week 1 and two times each at week 2.

2) Rheology

Rheology was performed on the Rheospectris optical rheometer. The samples were tested with frequency sweeps from 10 to 1000 kHz. An opaque coverslip was placed below the sample on the disk geometry sample holder in order to allow the sample to be easily identified by the Rheospectris. For each sample, the storage modulus was taken as the value at 1000 kHz.

III. RESULTS AND DISCUSSION

A. Pre-experiment

Preliminary experiments were performed to confirm the efficacy of HITED media to induce BMSC differentiation via expression of pan-keratin. Additionally, compaction of a BMSC-seeded collagen was measured in order to determine optimal seeding densities to mimic behaviors of fibroblast-seeded dermal equivalents.

1) Collagen Compaction

Collagen compaction was measured over the course of six days (Fig. 2) in order to determine the minimal seeding density that can mimic compaction behaviors of fibroblast-seeded dermal equivalents.

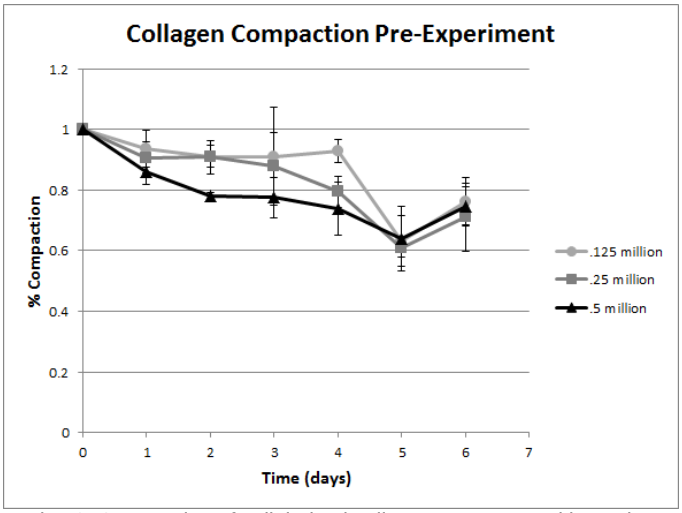

Fig.. 2: Compaction of cellularized collagen constructs with varying seed cell densities over one week. Varying densities were used to determine what seeding concentration most closely matched the behavior of fibroblast-seeded dermal equivalents, approximately 40% [11]. Higher levels of compaction were observed in constructs with higher seeding densities on days 1-4. However, on days 5 and 6, similar degrees of compaction were observed in all samples. As a result, 125,000 cells was chosen as the seeding density as the minimal concentration to achieve 40% compaction.

The general trend that all three samples followed matched that expected of collagen with cells mimicking fibroblasts. Until day 4, a trend based on seeding density is apparent, however, the samples follow the same trend for days 5-6. This pre-experiment allowed us to determine the optimal initial seed density for the complete experiment. Because after five days all conditions managed to achieve 40% compaction, which is also seen in fibroblast compaction experiments [11], we decided to seed with the lowest number of cells, 0.125 million cells per well. This ensured that the collagen would be adequately compacted by the time the BMSCs were seeded above the construct for differentiation at day 7.

2) Immunostaining for BMSC differentiation

Fig. 3 shows the morphology and pan-keratin expression in BMSCs cultured in HITED media after 4 and 10 days. Cells had primarily rounded morphologies, similar to keratinocytes in monolayer culture [2].This is distinct from BMSCs cultured in DMEM without differentiation factors, which have a more elongated morphology [2].

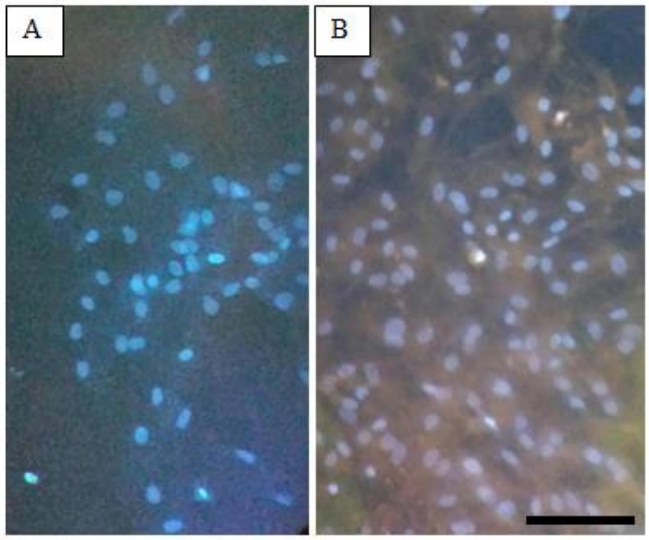

Fig. 3. Immunostaining of BMSC monolayer after 4 and 10 days cultured in HITED differentiation media. Nuclei stained with DAPI (blue) and cytokeratin expression with pan keratin-FITC (yellow).(A) BMSCs on the fourth day of the differentiation time course. (B) BMSCs on the tenth day of the differentiation time course. The increase in pan-keratin expression suggests that HITED has successfully induced a keratinocyte-like lineage in BMSCs. However, pan-keratin expression is moderately low, suggesting that these cells may still be in the early phase of differentiation. Scale bar is 5 microns.

In addition, pan-keratin expression was shown to increase between day 4 and day 10, suggesting that HITED has successfully induced a keratinocyte lineage in BMSCs. Expression was seen throughout the cell bodies, which is appropriate expression for an intracellular filament such as keratin [12]. However, the relatively weak cytokeratin expression implies that cells are still in the early differentiation phase.

B. Full Experiment

1) Immunohistochemistry of full skin constructs

To determine if our constructs became skin-like, we stained with DAPI and immunostained for pan-keratin (Fig.. 4b) and compared them to skin from a 7 day old fetal chick sample (Fig.. 4a).

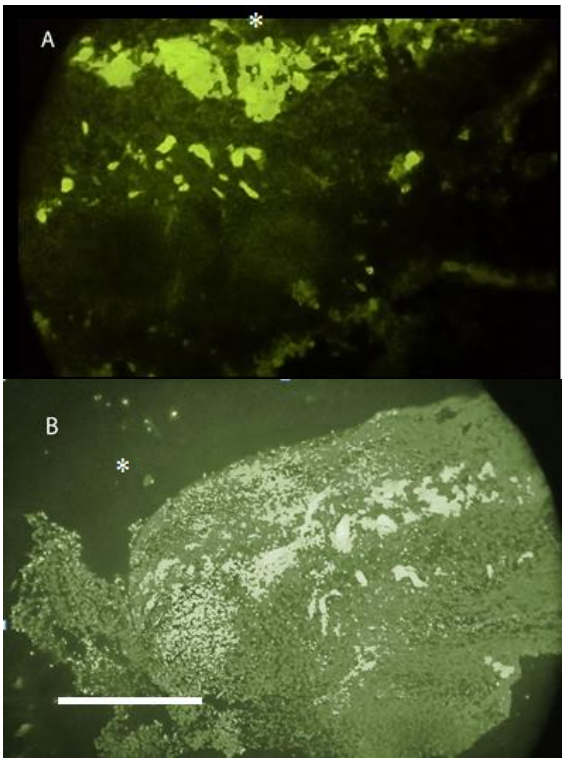

Fig.. 4: Histology of (a) 7 day old fetal chick sample and (b) control sample. Nuclei stained with DAPI and immunostained for pan-cytokeratin with FITC. Bright areas show expression of cytokeratin. Scale bar is 10 microns and asterisk represents the epidermal layer.

Staining of our control sample revealed a band of cytokeratin expression in the upper center, with potential stratification above the epidermal layer and the dermal layer. This appears to be similar to the expression patterns seen in the chick sample, suggesting that our construct is skin like in cytokeratin expression patterns. Additionally, regions of cytokeratin expression in the dermal layer may be explained by how the constructs were bottom fed and thus all cells were given differentiation media. That we see a band of prominent expression also points to how the air-liquid interface and surface-seeding of the BMSCs for differentiation are important in causing differentiation into keratinocyte-like cells.

2) Hardness

Hardness is the property that allows materials to resist plastic deformation such as penetration, indentation, and scratching. As skin is regularly exposed to similar mechanical loading, this test demonstrates this plastic deformation resistance of our skin is comparable to that of human skin [13]. The Shore 00 hardness test was used to measure hardness on the constructs.

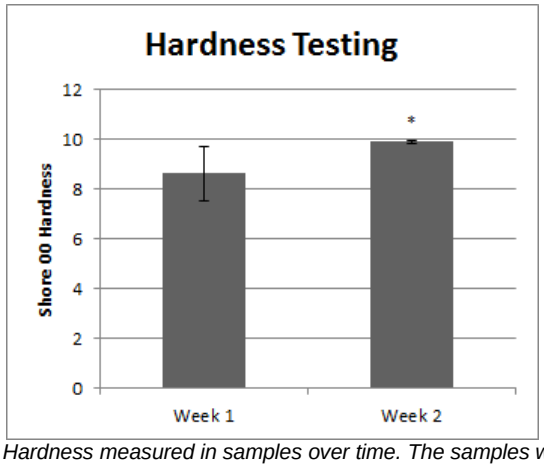

Fig. 5. Hardness measured in samples over time. The samples were measured for hardness over time with a shore 00 device. The samples became increasingly hard over time approaching the documented value for human skin hardness. T-test analysis confirmed that the week 2 samples were significantly harder than the week 1 samples ($p<0.1$).

Shore 00 hardness measures the softest materials. The tissues became harder over time as the cells compacted the collagen. After 1 week of culture, the hardness of skin constructs was 8.61±1.9 and after 2 weeks, it was 9.87±0.03 on the Shore 00 Hardness scale (Fig.. 5). The average hardness of human skin is approximately 10 [13]. As our sample hardness began to approach this value at week two, it suggests that our constructs were becoming more skin-like in their hardness. Week 1 samples were determined to be significantly different than week 2 samples using T-test analysis ($p<0.1$).

3) Toxicity

The goal of engineering skin as a model system is to create a system which is more accurate and ethical than animal models, and easier to obtain and culture than cadaver skin or neonatal foreskin models. One potential use of skin as a model system is to test drugs for skin toxicity or phototoxicity, and having an accurate and easy to use engineered skin model would be greatly beneficial to dermally-applied pharmaceutical research.

4) Histology

With the immunostaining, we expect to see colocalization of the FITC stain in the epidermal layer as the pan keratin stain will react to the microfilaments and stain cells that have cytokeratin filaments.

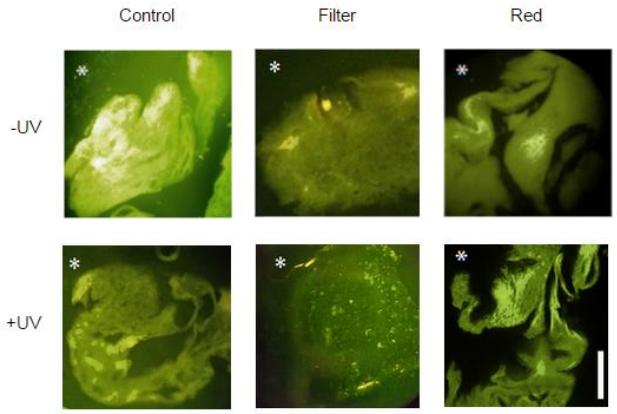

Fig. 6: UV and neutral red treated samples stained with DAPI and immunostained for pan-keratin with FITC. Control samples (no filter paper), filter paper control samples (filter paper with water rather than drug), and samples treated with neutral red at 0.01% on filter paper. Scale bar represents 10 microns and the asterisk indicates the epidermal layer.

Fig. 6 shows immunohistochemical images of pan-keratin expression and counterstained with DAPI. In all samples, the epidermal layer, indicated with the asterisk, has colocalized pankeratin-FITC stain (Fig. 6). Because the samples were fed from below, there was slight staining of cells in the dermal layer as well, which is seen in the upper three samples in Fig. 6. After the samples were treated with UV, there was slight degradation of the dermal layer, which caused epidermal sections to distribute throughout the sample as can be seen in the lower three samples in Fig. 6.

5) Rheology

Rheology is a test that measures the strain response to applied stresses. Skin undergoes significant applied stress over time, which has increased our interest in measuring the skin constructs response to stresses. Rheometer readouts values at 1000 kHz were used for comparison as shown in Fig. 7.

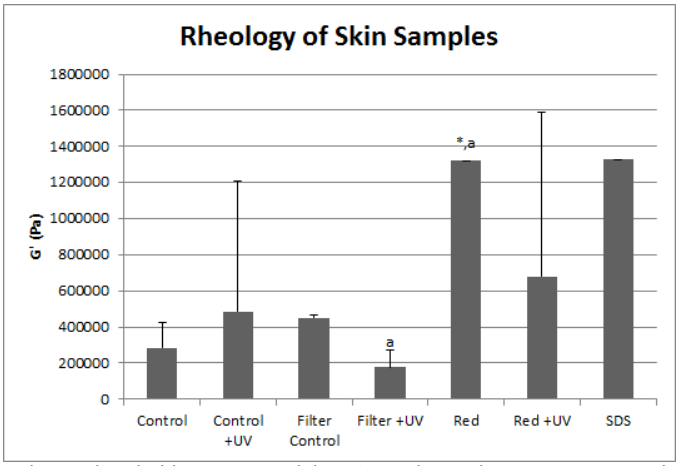

*Fig. 7. Threshold storage modulus. Control samples were not treated with any drug and did not have filter paper placed on them, filter samples had filter paper with water, rather than drug samples placed on them, red samples were treated with neutral red at 0.01% concentration on filter paper, and SDS samples were treated with SDS at 0.05% concentration on filter paper. A * denotes significantly different from control, and a from filter control (all T-test values p<.05). Also note that statistical analysis was not possible for SDS as only one sample was tested.*

These values demonstrate that the storage modulus, or the elastic response to applied stresses, change between samples. UV treatment was shown to significantly affect the filter samples, with the control significantly stiffer than the UV treated sample, as seen in Fig. 6. Treatment with neutral red (without UV treatment) appeared to make samples significantly stiffer ($p<0.05$) than the control and filter control samples ($p<0.005$). Additionally, only one SDS sample was usable for rheology, so although statistical analysis was not possible, if our reading was accurate, we would expect both SDS and SDS +UV (as SDS is not phototoxic) to have similar values and be significantly different from the control and filter paper samples. High variance from the optical testing method resulted in overall inconclusive data for determining effects of drugs or UV on construct stiffness. Additionally, the trend that sample stiffness goes up when treated with drugs is likely due to the drugs making the samples thinner and the frequency of the glass coverslip used to hold the samples becoming more prominent.

IV. Conclusion

Over the course of this experiment, we have demonstrated the ability to develop a skin construct that mimics human skin. The constructs are both morphologically and mechanically similar to human tissue as measured by histology, hardness, and rheology. Through these constructs, we demonstrate that BMSCs can be effectively differentiated to produce cytokeratin markers. Additionally, BMSCs can be used in the collagen cellular layer as fibroblasts. These tissue samples respond to both the drugs applied as well as UV treatment. Neutral red had a phototoxic effect on the sample, suggesting a BMSC differentiated model may be appropriate for testing phototoxicity of some drugs. From this work, we can conclude that the skin samples respond to drug testing.

Moving forward with this work, we would focus on improving the time course data, using better controls, and doing death staining of the drug and UV tests. Collecting more time points for hardness and histology testing would improve our ability to determine how different our skin construct is at initial seeding and after extended differentiation and compaction. Better controls, such as samples grown in enriched DMEM rather than differentiation media, would help us definitively demonstrate that our constructs have become more skin-like due to our efforts and not just due to the progression of time. Finally, death analysis and staining would allow us to better determine the toxicity of the UV and drugs

used. We would also need to ensure that the drugs would not dissolve the samples, potentially by using lower concentrations or different application methods. The field of engineered skin as a whole would benefit from more research into skin models for specific uses, such as drug phototoxicity, to allow for both more accurate and more ethical results than animal testing.

REFERENCES

[1] Asbill C *et al.* (2000). Evaluation of human bio-engineered skin equivalent for drug permeation studies. *Pharamceutical Research*, 17(9):1092-7.

[2] Ma K *et al.* (2009). Differentiation of bone marrow-derived mesenchymal stem cells into multi-layered epidermis-like cells in 3D organotypic coculture. *Biomaterials*, 30:3251-8.

[3] Singh, S., Zhao, K., & Singh, J. (2002). In vitro permeability and binding of hydrocarbons in pig ear and human abdominal skin. *Drug and chemical toxicology*, *25*(1), 83-92.

[4] Godin, B., & Touitou, E. (2007). Transdermal skin delivery: predictions for humans from in vivo, ex vivo and animal models. *Advanced drug delivery reviews*, *59*(11), 1152-1161.

[5] Scott, R. C., Walker, M., & Dugard, P. H. (1986). A comparison of the in vitro permeability properties of human and some laboratory animal skins.*International journal of cosmetic science*, *8*(4), 189-194.

[6] Schreiber, S., Mahmoud, A., Vuia, A., Rübbelke, M. K., Schmidt, E., Schaller, M., ... & Schäfer-Korting, M. (2005). Reconstructed epidermis versus human and animal skin in skin absorption studies. *Toxicology in vitro*, *19*(6), 813-822.

[7] Edwards, S. M. *et al*. (1994). Quantitative in vitro assessment of phototoxicity using a human skin model, Skin2. *Photodermaology Photoimmunology Photomedical*, 10(3):111-7.

[8] Gong and Niklason. Small-diameter human vessel wall engineered from bone marrow-derived mesenchymal stem cells (hMSCs). *FASEB J*. 2008 June ; 22(6): 1635–1648.

[9] Vaccariello M, Javaherian A, Parenteau N, Garlick J. Use of skin equivalent wound healing model. In: Morgan J, Yarmush M, editors. Tissue engineering methods and protocols. Totowa, New Jersey: Humana Press; 1999. p. 391–406.

[10] Liebsch *et al.* (1995). Application of the Human Dermal Model Skin2 ZK 1350 to Phototoxicity and Skin Corrosivity Testing. *Toxicology in Vitro,* 9(4):1557-562.

[11] Coulomb, B., Friteau, L., Baruch, J., Guilbaud, J., Chretien-Marquet, B., Glicenstein, J., ... & Dubertret, L. (1998). Advantage of the presence of living dermal fibroblasts within in vitro reconstructed skin for grafting in humans. *Plastic and reconstructive surgery*, 101(7), 1891-1903.

[12] Chu, P. G., & Weiss, L. M. (2002). Keratin expression in human tissues and neoplasms. *Histopathology*, 40(5):403-39.

[13] Kuwahara, Y., Shima, Y., Shirayama, D., Kawai, M., Hagihara, K., Hirano, T., ... & Kawase, I. (2008). Quantification of hardness, elasticity and viscosity of the skin of patients with systemic sclerosis using a novel sensing device (Vesmeter): a proposal for a new outcome measurement procedure.*Rheumatology*, *47*(7), 1018-1024.

V. APPENDIX 1: RHEOLOGY DATA

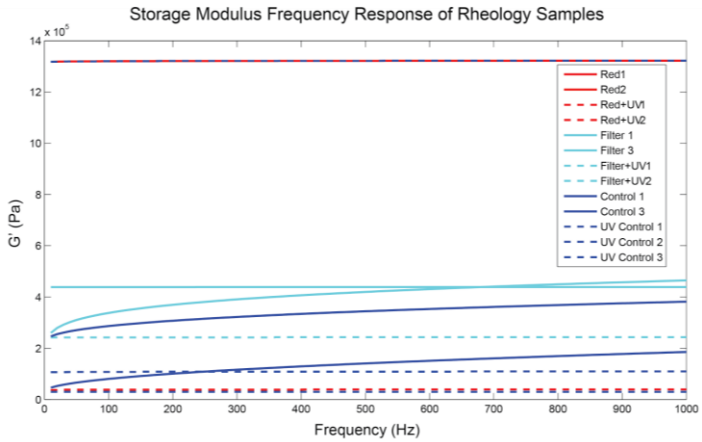

Fig. A1: Frequency response of storage modulus of rheology samples. SDS samples not shown due to deterioration of samples prior to testing