Self organization of Fibroblasts and Keratinocytes in morphogenesis of dermal-epidermal junction in human skin equivalents

Chandra, P.\(^1\), Rai, V.\(^2\), Michniak-Kohn, B.\(^{2,1}\) and Kohn, J.\(^1\)

\(^{1}\) New Jersey Center for Biomaterials, Piscataway, NJ, USA
\(^{2}\) Ernest Mario School of Pharmacy, Rutgers – The State University of New Jersey, Piscataway, NJ, USA

Introduction
Dermal epidermal junction (DEJ) is a complex macromolecular structure which establishes the boundary between two major skin compartments, epidermis and dermis and the cellular components of these layers play a major role in its organization (1). We are interested in the structural and functional analysis of the epidermis formed in human skin equivalent (HSE) models that uses co-cultures of fibroblasts & keratinocytes (Three-dimensional organotypic co-cultures) to derive the epidermal layer. Self-organizing capabilities and live cell fluorescence imaging of these two cell types in co-cultures on three-dimensional tissue constructs (dermal layer) will be used to determining the composition and conditions for the co-cultures that form epidermis structurally and functional similar to those in human skin.

Methods
Live Human dermal fibroblasts (HDF) & Human keratinocytes (HK) cells (Cascade Biologics, Portland, OR, USA) were labeled using CellTracker Green CMFDA; Ex/Em 492/517 nm) and CellTracker Red CMTPX; Ex/Em 577/602 nm) (Invitrogen Corp, Carlsbad, USA) and fluorescence and viability was followed for 5 days post staining. Image analysis was used to determine residual fluorescence at different times in the two cell types separately. Two Human skin equivalents models (one consisting of collagen + fibroblast only dermis and the other polymer scaffold + collagen + fibroblasts containing dermis) were cultured and the epidermal layer formation was evaluated using different ratios of keratinocytes & fibroblasts (K:F). An optimal ratio of K:F is being used to construct the epidermis in HSE models using fluorescently labeled cell co-cultures.

Results
Live cell fluorescence labeling of human fibroblasts & keratinocytes show a concentration dependent effect of cell tracker dyes. CellTracker Green CMFDA was found suitable for labeling fibroblasts (optimal concentration of 2.5 μM) and CellTracker Red CMTPX for the keratinocytes (2.5 μM) for at least 5 days of culture post labeling (Figure 1). Culture of full thickness human skin equivalents (HSE) with keratinocytes & fibroblasts (K:F) ratio of 5:1 showed a better formed epidermis compared to K:F 5:5 (Figure 2). Epidermal morphogenesis in HSE’s being developed in this laboratory is being studied using fluorescently labeled K:F co-cultures and later analysis using immunostaining of prominent differentiation markers.

Figure 1. Live cell labeling of fibroblasts (A) with CellTracker Green CMFDA and Keratinocytes (B) with CellTracker Red CMTPX. Co-cultures of labeled keratinocytes & fibroblasts will help track cell fates in epidermal morphogenesis during HSE culture.

Figure 2. Epidermis (Arrow) and superficial dermis of HSE’s made using collagen & fibroblasts + keratinocytes (A), Electrospun poly(DTE carbonate) scaffold, collagen & fibroblasts + K:F (5:1) (B) and Polymer scaffold, collagen & fibroblasts + K:F (5:5) (C). Tissue in C has less thickness (150μm) than A & B (400-360 μm).

References
Electrospinning of polymer scaffolds for skin tissue engineering

Rai, V.2, Chandra, P.1, Florek, C.1, Michniak-Kohn, B.2,1 and Kohn, J.1

1 New Jersey Center for Biomaterials, Piscataway, NJ, USA
2 Ernest Mario School of Pharmacy, Rutgers – The State University of New Jersey, Piscataway, NJ, USA

Introduction
Electrospinning is a technique that enables fabrication of 3D nanofibrous scaffolds for tissue engineering applications as they closely mimic natural extracellular matrix (1). We are using a biocompatible synthetic polymer poly(DTE carbonate) to electrospin 3D fiber mats to be used as dermal scaffolds making human skin equivalents (HSE), which will have improved mechanical properties and better handling during the culture periods and in vitro applications compared to currently available models. HSE’s are promising for several ex vivo applications, including screening platforms for drug delivery, toxicology, skin irritation and inflammation, skin biology research; as well as for skin tissue replacement therapy.

Methods
A 26% wt/vol solution of the polymer poly(DTE carbonate) in THF/DMF was electrospun to generate fibroporous scaffold of required thickness. The scaffold was imaged using scanning electron microscopy. Neonatal Human Dermal Fibroblasts (HDF) attachment and proliferation in the scaffold was measured using the MTS assay (2), Fluorescence staining/Microscopy and Image analysis (ImageJ). Dermal and Full-thickness skin layers were prepared and tissue histology was analyzed using cryosectioning, Hematoxylin & Eosine (H&E) staining, Fluorescence staining and Image analysis.

Results
Highly porous electrospun scaffolds, with fiber diameter ranging from 3-5 µm and pore-sizes were ranging from 50-100 µm, was prepared (Figure 1A) and was shown to allow human dermal fibroblasts (HDF) cell infiltration and attachment inside the scaffold. Dry cell-free scaffolds had tensile strengths of around 75 MPa, and the presence of the electrospun scaffold could also reduce the contraction of the collagen-only based dermis by about 80%. The histology of the electrospun polymer containing dermal tissues shows that the fiber mat creates a better integrated tissue, where the dermal fibroblast cells populate the entire tissue (Light blue cell nuclei staining in Figures 1B). Full thickness HSE are being constructed using the electrospun scaffold (example in Figure 1C), which will be analyzed for tissue formation using histology, immunohistochemistry and permeability characteristics will be evaluated and compared to other available HSE models and the human skin.

![Electrospun poly(DTE carbonate) scaffold](image1)

![Dermal layer containing electrospun polymer](image2)

![HSE culture](image3)

**Figure 1 (A).** Scanning Electron Micrograph of the electrospun polymeric scaffolds at 150X. (B) Histological analysis of the dermal tissues at Day 7 made using electrospun polymer and collagen. Blue fluorescence from staining the cellular nuclei using Hoechst. Scale bar 100 µm. (C) Dermal layer of HSE containing electrospun poly(DTE carbonate) scaffold (circled) growing in culture.

References
**Introduction:**
Biodegradable tyrosine-based self assembling nanospheres are promising systems for drug delivery. Recent studies demonstrate that these ABA-triblock copolymers consisting of long poly (ethylene glycol)–PEG A-blocks and polyaryl B-blocks strongly interact with hydrophobic drugs\(^1\). To better understand the binding affinity of several drugs with these polymers, we developed a computational method that combines Molecular Dynamics (MD) simulations and docking studies. Desaminotyrosyl–tyrosine octyl ester suberate (DTO-SA-PEG/5K) was selected as the first polymer, and curcumin and paclitaxel as candidate drugs, for which experimental data are available. Preliminary results demonstrate the feasibility of this approach.

**Methods:**
First, the 3D structure of DTO-SA-PEG/5K polymer was relaxed in water by performing Molecular Dynamic (MD) simulations using MOE (Molecular Operating Environment) software package. Next, each of the two drugs were docked (Autodock4 software) in the core (B-block), DTO-SA. A grid refinement study was performed for the docking calculations to insure convergence of the computed expected value of the binding energy.

**Results:**
The calculated expected values of binding energy for curcumin and paclitaxel are \(-6.2\pm0.2\) and \(-4.6\pm0.3\) kcal/mol, respectively, thereby indicating that curcumin binds more tightly to DTO-SA-PEG/5K. Recent experiments at the New Jersey Center for Biomaterials confirm this finding: binding efficiency of 94% and 66% for curcumin and paclitaxel.

**Conclusion:**
A model for predicting binding of drugs to polymeric nanospheres is developed based upon Molecular Dynamics simulations and docking calculations. The model predicts the observed relative binding affinity of two different drugs to DTO-SA-PEG/5K. Current research focuses on extending the model to additional drugs and polymers.

**References:**
Introduction: The intensive labor and high cost of developing new biomaterials highlights the need for a change in their discovery process. Predictive computational models can be used to accelerate the selection process of lead compounds from large polymers libraries for synthesis and biological characterization. For this purpose, the “Biomaterials Store” -- a computational tool for development of new biomaterials that integrates a database of large polymer libraries and modeling techniques -- has been developed at the New Jersey Center of Biomaterials (NJCBM). In this study we present an application of this computational tool for predicting the behavior of proteins in Hydrophobic Interaction Chromatography (HIC) systems, an experimental technique for separation or purification of proteins. The present study reconsiders the experimental data of Ladiwala et. al.\(^1\) using a different approach to predict the protein retention time based on a hybrid Decision Tree (DT) and Artificial Neural Network (ANN) model and traditional 2D and 3D protein descriptors. The objective of this work is to investigate the performance and sensitivity of our statistical methods using the “Biomaterials Store” and compare with previous work\(^1\).

Methods: The models for 27 protein and four polymer resins were developed using “Biomaterials Store”, based on the Waikato Environment for Knowledge Analysis (WEKA) environment integrated with a MySQL database. The computational steps for the development of the models are shown in Fig. 1.

Results: The experimental protein retention time\(^1\) for 27 proteins was used with 50% of the data assigned randomly to the training set and 50% to the validation set. Models were developed for each of the four polymer resins. The results for the training set show very high correlation coefficients with most frequent values between 0.9 and 1.0, thereby indicating that the ANN is able to build a model for the training set based upon the descriptors selected by the C4.5 Decision Tree. The Pearson correlation coefficient for the validation set ranges from 0.3 to 0.4 which represents a predictive model of reasonable accuracy. These findings demonstrate that descriptor set selection is of critical importance for enhancing the predictive capabilities of these types of models, with descriptors that can most closely capture the fundamental governing factors that represent the specific intermolecular interactions involved providing the greatest degree of correlation. This study further suggests that the inclusion of descriptors characterizing the 2D and 3D nature of the polymer surface itself, which have not been represented in either our study or the previous study by Ladiwala et al\(^1\), may lead to further improvements in the ability to predict the retention and selectivity of proteins for these types of chromatography systems.

References:
The design of biologically responsive materials for regenerative medicine depends on understanding the interactions between the material and the extracellular matrix (ECM). Among the factors that affect cellular responses, the chemistry of the material, the mechanical stiffness and the presence of adhesive ligands on the biomaterial are known to have considerable effects. We are employing a library of methacrylate polymers to understand the effects of chemistry, mechanical stiffness and adhesivity on cellular functions.

In this work we synthesized a library of hydrophilic polymers with low modulus whose mechanical properties can be varied independently of their adhesive properties. Each polymer is synthesized from three monomers by AIBN mediated radical polymerization. The mechanical stiffness of each polymer is determined by the feed ratio of the monomers. The polymers were then surface modified with RGD peptides to provide specific cell adhesivity. This design provides the flexibility of independently varying the chemistry, stiffness and adhesivity of each polymer.

Assessment of cellular functions was evaluated on polymers coated on glass cover slips. 3T3 cell proliferation was influenced by both the chemistry and stiffness of the material although the chemistry of the material had a greater influence than the stiffness. Subsequently surface functionalization of the polymers with RGD peptides increased the proliferation of 3T3 cells. hMSC differentiation on the library of polymers was also evaluated as a function of chemistry, stiffness and adhesivity of the polymers. It was seen that the expression of alkaline phosphatase was greatly influenced by the chemistry of the material. Currently detailed studies are in progress to evaluate the expression of various differentiation markers on these polymers. Through our studies we expect to achieve a better understanding of the interactions between cells and the materials that they interact with, which would enable the development of interactive biomaterials for regenerative medicine.
Cancer therapy necessitates surgical resection of malignant tissues with consequent tissue regeneration, which may be accomplished by implanting biomaterial scaffolds containing or attracting autologous stem cells, such as mesenchymal stem cells (MSCs). However, this approach requires further development, as recent studies demonstrate that MSCs can become malignant due to abnormal signaling from interactions with cancerous microenvironments. Indeed, cancer recurrence is shown to derive from a subpopulation of stem cells. It is therefore desirable to design biomaterials that prevent abnormal MSC transformation, while preserving strategic differentiation capabilities to regenerate tissues damaged from the surgical resection. There are also increasing needs to develop reliable methods enabling an early prediction of malignant cell transformation. To meet the challenges, we developed an in vitro carcinogenic model inducing aberrant MSC transformation by nickel sulphate. We analysed spatial distribution patterns of nuclear mitotic apparatus proteins (NuMA)s and subsequent changes in nuclear morphometric parameters (descriptors) in normal vs. aberrant MSCs using multiphoton imaging-based screening and quantitative analytical tools. We studied correlations between the changes of nuclear morphometric parameters and the degrees of aberrant MSCs transformation. In parallel, to identify bioactive components influencing MSC transformation, we used tyrosine-derived polycarbonates, copolymerized in selected molar ratios of (i) lipophilic monomers, (ii) hydrophilic monomers, poly(ethylene glycol) (PEG), and (iii) negatively charged monomers (DT). We indentified very useful nuclear descriptors to predict aberrant MSC transformation, such as margination and roundness. Moreover, we found DT, as opposed to PEG, to both promote MSC growth and reduce abnormal transformation. Our results suggest a new concept and method for biomaterial design in regenerative cancer therapy.
An Improved TIGER Sampling Method for Large Systems to Perform Replica Exchange with the Number of Replicas and Temperature Levels Set Independent of System Size

Li, X.1, Latour, R.A.1, Stuart, S. J.2
1Department of Bioengineering, Clemson University, Clemson, SC
2Department of Chemistry, Clemson University, Clemson, SC

A revised advanced sampling method for molecular simulation based on “temperature intervals with global exchange of replicas” (TIGER2) was developed to reduce the high demand of computational resources and the low computational efficiency of the conventional replica-exchange molecular dynamics (REMD) method, which uses elevated temperature to facilitate sampling. This new method overcomes the limitation of its previous version (Li et al., J. Chem. Phys., 2007), which requires the assumption of constant heat capacity during quenching of replicas from elevated temperatures to the target temperature. The analytical solution of TIGER2 for a two-state simple model proves that the algorithm rigorously satisfies the detailed balance condition when the quenched replicas provide a random sampling of states. The robustness of the TIGER2 method is examined by sampling the conformation distribution of a single butane molecule in vacuum and a model peptide of (AAQAA)3 in aqueous solution. The agreement between the REMD and TIGER2 results for these systems indicates that the TIGER2 algorithm satisfies the detailed balance condition and is able to build proper Boltzmann-weighted ensemble of states. TIGER2 should substantially enhance our abilities to conduct simulations of large, complex systems, such as bulk polymers, polymer surfaces, and protein-polymer interactions.
A systematic procedure has been developed to coarse grain an atomistic amorphous polymer model into a mesoscopic model and this method has been used to develop a coarse-grained model of DTB succinate, a representative tyrosine-derived polyarylate. This method is based on an iterative algorithm for potential inversion from distribution functions of the atomistic model. Both static and dynamic properties from the atomistic and the coarse-grained simulations have been analyzed and compared. The coarse-grained model, which contains the essentials of the DTB succinate structure, successfully described the total chain geometry. The effective speedup compared to the corresponding atomistic simulation is significantly above 10^3, and thus the simulation times reach well into the characteristic experimental regime. These models should enable us to develop accurate simulations of amorphous polymers, which can then be used to study and predict events at the molecular level that are relevant for biomaterials applications, such as the absorption, diffusion, and clustering of water within the polymer, and investigating how polymer structure influences the bioactive state of adsorbed proteins and subsequent cellular response.
Controlling the protein adsorption process on biomaterial surfaces is an important challenge for biomedical engineering. The performance of a medical device may be linked to the way proteins adsorb to its surface once it is implanted. It has been well documented that in random copolymer systems composed of hydrophobic units and PEG blocks, increasing the amount of PEG reduces protein adsorption and subsequent cell attachment. However, it is currently poorly understood how variations in the PEG block size affect polymer morphology and the subsequent protein adsorption process. To address this question, we prepared a system of three model polymers comprised of a hydrophobic monomer (desaminotyrosyl-tyrosine ethyl ester, DTE) which was copolymerized with PEG blocks of different molecular weights (100, 1000 and 35,000). In all polymers, we kept the total weight percentage of PEG constant at 40 weight %. Therefore, the only difference between these polymers was the PEG block length and the associated changes in polymer morphology. As expected for a PEG-rich polymer, we find that fibrinogen is repelled when the PEG block length is 1000. However, when PEG_{100} and PEG_{35k} were used, fibrinogen adsorbs to the corresponding polymer surfaces readily. Analysis of the bulk materials using small angle neutron scattering (SANS) indicates that the copolymer with PEG_{35k} blocks contains PEG domains with radii of about 10 nm separated by 33 nm between domains. In contrast, the copolymer with PEG_{1000} blocks had PEG domains with radii of about 5 nm separated by 15 nm between domains. Although the polymer containing PEG_{100} could have PEG-rich domains separated by > 70 nm, a distance that was at the limit of instrument resolution, it is most likely that PEG is homogeneously distributed at ~ 1 nm length scales. Since the dimensions of a molecule of fibrinogen are 47 nm x 4.5 nm x 4.5 nm, we speculate that the modulation of protein adsorption is caused by the spatial distribution of PEG domains: If PEG domains are sufficiently far apart, proteins will adsorb onto the hydrophobic DTE regions. This condition was met only for copolymers having PEG_{35k} and PEG_{100} blocks. The data presented provide new insights into the mechanisms of surface-protein interactions and how these interactions can aid the rational design of biomaterials for tissue engineering.