Bio Roll-Up: Self-Assembling Scaffolds For Tissue Engineering

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Abstract

There is an unmet need for controlled, dynamic cell scaffolds that will generate 3-D monolayers of epithelial cells for medical research and tissue engineering. Here we describe a system that uses photoimageable and biocompatible HEMA-based hydrogel bilayers that self-assemble from 2-D substrates into 3-D tube-shaped structures. These structures are patterned on top of another layer that demonstrates an ability to anchor the assembled structure and control the direction and extent of self-assembly. Cell viability was determined by MTT assay which shows material components comparable with the positive control (80-110%). Finally, the masks used in the experiments herein were redesigned to improve ease of use, alignment and testability.
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Background

**Photoresists** A photoresist or, simply resist, is a light-sensitive material that can be imaged and developed to form almost any pattern or shape. The resists described herein are composed of organic polymers and other molecules coated on a substrate which can be silicon or an organic layer called an underlayer. Upon exposure to UV light, chemical changes take place within the resist (Figure 1). This alters the polymers solubility in a developer. Positive resists become more soluble in areas of UV exposure whereas negative resists crosslink (Figure 2) to become less soluble. After development of negative-tone resists, the unexposed regions of film dissolve away and the desired pattern remains.

**Hydrogels** A hydrogel is an absorbent material where a lightly-crosslinked polymer network uses water as its dispersive medium. Hydrogels are an important class of materials in the medical sciences. They are used in wide-ranging applications such as contact lenses, drug delivery and cellular scaffolds in regenerative medicine. Hydrogel engineering has reached a sophisticated level of development giving engineers the opportunity to precisely control physical and chemical properties in order to manipulate biocompatibility, biodegradability, elasticity, etc.1

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**Figure 1.** A photoresist is a light-sensitive material that is typically coated on a wafer of silicon or an underlayer (not shown). A photomask is used in combination with a light source to expose certain areas of the photoresist. The exposed areas change chemically to be either more or less soluble in a developing solution (called the developer.) Upon development of a positive-tone resist, exposed areas dissolve. When negative-tone resists are developed, the unexposed areas dissolve away.
Introduction

Diseased, cancerous or damaged tissues must be removed from a patient's body and replaced with new tissue when conventional therapies fail. The ultimate goal of tissue engineering is to replace entire human organs; however, there are still many tissues too complex to fabricate with contemporary techniques. Tissue engineers deploy a variety of techniques to create three-dimensional (3-D) tissues, such as micromolding and bioprinting, in order to form dense aggregates of cells. The adaption of 3D-printers for biological applications has advanced considerably in recent years, but still faces several challenges that prevent wide-spread adoption. Not the least of which is the considerable cost for these systems. More common techniques are sufficient in the generation of liver, heart or bone tissues, but many tissue types still cannot be generated in this manner. Tissues such as glands and ducts are composed of epithelial cells in 3-D monolayers—not as aggregates of cells (Figure 3). Examples of these include salivary glands, kidney tubules, lung bronchioles and mammary glands. New scientific and engineering advances are needed to give tissue engineers the ability to construct these tissues.

The Bio Roll-Up Project seeks to develop a new process for producing and studying 3-D monolayers of epithelial cells for use in basic, clinical and applied research. This technique incorporates a stack of biocompatible hydrogels that will self-assemble into 3-D scaffolds for cell growth (Figure 4). By seeding epithelial cells onto the hydrogel stack, followed by self-assembly, 2-D planar sheets of cells will be converted into 3-D monolayers. Developing this process would give tissue engineers the ability to grow tissues and organs that cannot be made with contemporary techniques. It would also broaden scientific understanding of the affect hydrogels have on cell biology. For certain cell types, it has been shown that cell morphology can be affected by the shape of the substrate. However, there is limited information published on the effect of substrate shape on the determination and differentiation of
epithelial cells. If tissue engineers are to produce tissues which contain curved motifs, such as in salivary glands, a better understanding of how a cell’s microenvironment can affect differentiation is necessary. A dynamic and tuneable scaffold would enhance the study of cell differentiation by controlling variables such as scaffold elasticity, surface chemistry, self-assembly kinetics and substrate curvature.

To produce a highly tunable scaffold, the Bio Roll-Up system consisting of four main components is employed. First, a “slippery” underlayer is coated onto a silicon wafer. This underlayer allows the scaffold to leave its surface. Second, a negative-tone “Sticky Layer” (SL) is patterned on top of the underlayer. The SL ‘sticks’ to both the underlayer and scaffold. The Sticky Layer has two elements: a thick “sticky strip” that permanently adheres to the scaffold structure and micro-sized ‘islands’ called “speed bumps” that, by manipulating size and density, provide temporary adhesion to the scaffold. The last two components are the Bio Layers (BL). They are each composed of a polymer precursor that forms a hydrogel upon exposure to UV light. One advantage of using this type of so-called “post-synthetic crosslinking” is the control it provides over crosslink density. Once the resist stack is synthesized, it will be sterilized and introduced into a cell medium where epithelial cells will be seeded on the top layer, Bio Layer 2 (Figure 4). Overtime, (1-24 hours) it will self-assemble into the desired structure. The driving force behind self-assembly is the differential swelling stress between Bio Layer 1 (BL1) and Bio Layer 2 (BL2). Bio Layer 1 possesses a higher swelling ratio than BL2 due to its lower crosslink density. When the two layers swell, a stress is introduced at the interface which is relieved through bending or rolling up.

![Figure 3](image)

**Figure 3.** Left: The diagram above shows a (A) salivary gland composed of three target structures for this novel self-assembly process: (B) Tubes, (C) intersections and (D) spheres. Right: If two hydrogels of different swelling ratios are attached, they can induce bending to form three-dimensional structures such as tubes and spheres.
A significant amount of literature exists describing the self-assembly of various tissue scaffold materials and designs. Yuan et al. used a single layer of strained PDMS as a mechanism to achieve a rolling structure. Other groups seek to use highly biocompatible protein-based materials as cellular scaffolds. Both of these approaches generally lack in mechanical and chemical flexibility. Groups that utilize hydrogel bilayers as scaffold material provide important theory on the mechanics and behavior of bilayers; however, many of these studies use stimuli-responsive hydrogels that would be inappropriate for use in the constant temperature and pH environments of cell media. Many of the materials described are highly absorbent and biocompatible, but lack the mechanical and chemical manipulation needed for more advanced tissue scaffolds. The Bio Roll-Up project seeks to use hydroxyethyl methacrylate (HEMA) copolymers as BL polymer precursors. Copolymers of HEMA are of particular interest to tissue engineers due to their ability to form hydrogels with wide-ranging mechanical properties and biocompatibility.

While hydrogel bilayers have been used in the past as candidates for tissue scaffolds, none suggest full kinetic control over self-assembly and generally rely on changing materials—an internal change—to alter kinetics. Internally changing scaffold materials to control kinetics has a number of disadvantages by simultaneously altering other properties such as biocompatibility (swelling thermodynamics, surface charge density, functional group density, etc.), viscoelastic properties, porosity to facilitate diffusive nutrient transport or vascularization or biological/chemical degradation. An
alternative is the development of external methods of controlling self-assembly that can leave tissue engineers free to use the scaffold best suited for their cell types. The SL uses physical or chemical methods for controlled release of the BL scaffold.

**Previous Design** Self-assembly was first demonstrated using an alternative system to the current version. The previous system had three components: a positive-tone release layer (RL) and two BL’s. The RL was a sacrificial layer and provided kinetic control of self-assembly by dissolving away to gradually ‘release’ BL1 (Figure 5). The BL polymer precursor consisted of HEMA copolymerized with dimethylaminoethyl methacrylate (DMAEMA). Bio Layer self-assembly only occurred in highly acidic water (pH ~4). The materials were also not tested for compatibility in a biological system. Replacement of the RL with the negative-tone SL addressed several challenges and had several potential advantages. Those challenges included: Release layer solubility was being affected by BL coating and imaging, casting solvent constraints placed upon BL1, RL monomers must be regularly synthesized, etc. Shifting to the Sticky Layer solved most of these issues. The simpler system also came with important advantages: the absence of active dissolution of chemicals into the swelling solution, components are easy to obtain and synthesize and gives BL1 a greater degree of material and process flexibility by replacing it as the scaffold anchor. The BL polymer was also changed from poly(HEMA-co-DMAEMA) to poly(HEMA-co-Acrylic Acid) to avoid possible biocompatibility issues from the degradation of DMAEMA and to increase the hydrophilicity of the BL polymers.

![Figure 5](image-url)

*Figure 5.* (A) Release layer (RL) and two Bio Layers (BLs) are patterned on a silicon wafer. (B) The stack is immersed in cell buffer and seeded with cells. (C) Over the period of 1-24 hours, the RL dissolves to allow the BL’s to roll into a tube (D).
Results and Discussion

Early versions of the Bio Roll-Up system functioned only on virgin silicon. An organic underlayer was adapted to provide a more cost-effective and adaptable substrate. A variety of underlayer polymers were sampled. The formulations were used in the production scheme depicted in Figure 6. The underlayers were rated by the percentage of bilayers that self-assembled (SAR, self-assembly ratio) where an ideal “slippery” substrate would provide a SAR of 100%. Underlayers formulated with poly(HEMA-co-AA) possessed the highest ratio. Further experiments tested the effect of crosslinker load and bake time on the SAR. Resist bilayers were spin coated and imaged on each underlayer followed by salt impregnation and DI water soak. The SAR was taken and added to Table 1. Increasing bake time seemed to be proportional to SAR. The underlayer that had an acid crosslinker load of 25% and a bake time of 120 seconds is shown with self-assembled structures in Figure 7 and was used as the underlayer standard for the rest of the experiments.

Figure 6. A brief summary of the process steps required to synthesize BL1 and BL2 on the underlayer. First, the underlayer is spin coated and cured at 200°C. Both BLs are spin coated and exposed together to better define their shape and avoid alignment issues.
Table 1. Eight underlayer formulations were investigated using poly(HEMA-co-AA). The highest self-assembly ratio (SAR) was seen in underlayer formulations incorporating a crosslinker load of 25%.

<table>
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<tr>
<th>Crosslinker Load (%)</th>
<th>Bake Time (sec)</th>
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<td>20%</td>
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<td>50%</td>
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In a typical process (Figure 8), underlayer is spin coated on a 100 mm silicon wafer and baked on a hot plate \((t = 63 \text{ nm}, \sigma = 4 \text{ nm})\). Rectangular bilayers of negative-tone Bio Layer photoresists are coated \((t = 4.54 \mu m, \sigma = 0.15 \mu m)\) and imaged on the previously described underlayer. The bottom and top resist layer is Bio Layer 1 and Bio Layer 2, respectively. After the sheets are soaked in PBS and dried with flowing nitrogen, they are immersed in DI water. The bilayer sheets self-assemble into tubes and “scrolls” (sheets that rolled up from two edges) over 10-100 seconds. Tube alignment is biased towards initial resist pattern orientation however, mechanically perturbed tubes orient and move randomly laterally and orthogonally to the substrate (Figure 7, A). Long-edge rolling was almost exclusively observed. Higher resolution optical images of tubes show that tube diameter overlaps with target values \((40-200 \mu m)\) and that multiple rolls—or “spiraling”—occurs which give the tubes two or three ‘walls.’

In order to control spiraling and tube diameter, the effects of crosslinker load must be studied more closely. Changing the crosslinker concentration affects the concentration of the other components. Based upon profilometer data, altering the polymer concentration (the primary constituent of the film) significantly affects film thickness. It has been shown that spiraling and tube diameter is dependent on relative film thickness.\(^7\) It would be desirable to change crosslinking loads without affecting film thickness. Due to its similarity in solubility and structure, low molecular weight PEG chains (1-1.5 kDa) could act as a non-reactive substitute for the PEG-based crosslinker used in the Bio Layer formulations. By adding PEG, the polymer and initiator concentrations remain fixed while changing the crosslinker load.
The required impregnation of salt into the film indicates that the hydrogels lack the osmotic pressure required to swell in high-salt solutions. As of yet, self-assembly of these tube structures only occurs significantly in DI water. There are several methods for enhancing the osmotic pressure of hydrogel films. In general, adding charged co-monomers with mobile counter-ions is a common method of increasing osmotic pressure. This is consisted with our experiments whereby the addition of salt inside of or onto the hydrogels achieved self-assembly. It also suggests that increasing the AA monomer concentration in the polymer will increase swelling in buffer solutions. Indeed, moving to higher AA-containing polymers have allows us to achieve SAR of 15-20% in PBS; however, more work must be done. It was also found by Thienen et al. that the presence of free, low molecular weight (<10 kDa) poly(ethylene glycol) (PEG) chains in a degrading PEG-HEMA hydrogel significantly increased the osmotic pressure of the gel. These sources suggest that additives in the Bio layer formulations could allow for enhanced swelling in buffers with large osmotic pressures. An aqueous casting solvent must be present for salt additives. It was found that 0.2% (w/w) NaCl solutions are soluble in 95%/5% PM/DI water (dissolving in water first). It is worth noting that while water is generally not used as a casting solvent, polymers with high acrylic acid content dissolved in PBS will spin coat on the UL. This suggests that a move towards aqueous solvents is possible; however, free PEG chains should be first investigated due to its solubility in PM.

Development of the Sticky Layer needed to ensure that it adhered to both the underlayer and Bio Layers. Two negative-tone resists were made with the chosen polymer, poly(HEMA-co-MMA). Both were formulated with a photo-acid-generator (PAG) and an acid-catalyzed crosslinker while one had an

Figure 7. Bio layers imaged on underlayer without Sticky Layer. (A) Low resolution image of ~88 Bio Layer tubes. (B) 20X image of a tube showing several rolls, a phenomenon called “spiraling.” (C) Image of Bio Layers assembling from two sides creating a “scroll.” (D) Spiraling is shown on all tube or scroll structures.
additional, latent free-radical crosslinker (10% w/w; polymer conc. 75% w/w). It was hypothesized that latent radical crosslinkers in the Sticky Layer would be able to form covalent bonds to radical crosslinkers in the Bio Layers upon Bio Layer imaging. Adhesion to the underlayer was assessed by imaging the Sticky Layer on the underlayer and continuing process steps (imaging, development, etc.) without spin coating the Bio Layers themselves. These steps were followed with both Sticky Layer formulations and both showed no signs of delamination (Figure 8, B). In order to investigate adhesion to the Bio Layers (even under strain caused by self-assembly), Sticky Layer was spin coated ($t = 246$ nm, $\sigma = 19$ nm) and flood exposed (exposed without photomask) and Bio Layers were patterned on top (Figure 8, A). The average SAR after two minutes was less than 10% for both formulations and therefore no significant differences were shown.

![Figure 8.](image)

Integration of the Sticky Layer into the full Bio Roll-Up system looked at three aspects of the Sticky Layer interaction with the Bio Layer self-assembly: sticky strip effectiveness as an anchor for the scaffold structure, its ability to control assembly direction and speed bump density effect on assembly time or extent. Wafer samples were fabricated with all layers according to the Bio Roll-Up design (Figure 9). The samples were then soaked in PBS and dried under flowing nitrogen. Each sample was then immersed in DI water and optically imaged.
The Sticky Layer successfully anchored assembled structures against mechanical preturbations at a success rate of 90%. However, incomplete self-assembly was common (Figure 10) and is likely connected to Sticky Layer development. It was observed that development time affected the underlayer surface in such a way as to reduce the SAR. Increased development time would cause water to bead more readily suggesting a more hydrophobic surface. This could cause uneven drying after the PBS soak step leading to reduced SAR. The strip has also been shown to successfully control assembly direction by overcoming the Bio Layer’s natural tendency to engage in long-edge rolling (Figure 11).

Varying speed bump density demonstrated the ability to affect the extent of assembly (Figure 12). Increasing speed bump density coresponded with a decrease in assembly extent. There is little evidence to suggest that the speed bump size (≥ 625 μm²) and density used increased assembly time to target duration (~1 hour). Rather assembly showed on/off characteristics. This is likely due to the mask probing only large speed bump sizes and high densities up to 90% area coverage under the Bio Layers.

Figure 9: Above is a brief description summarizing the lithographic process used to create the complete Bio Roll-Up System.

Figure 10: Bio Layers imaged on sticky strip. (Scale bar = 0.1 mm)
An MTT assay was performed using mouse epithelial stem cells. The results are displayed below. It was shown that cell viability remained comparable to the control for all unpurified materials (Figure 13). While the MTT assay should remain a general test for biocompatibility, a more detailed biological study on the BRU materials is required for use as tissue scaffolds. For instance, adequate nutrient and waste transport is a challenge for cells in complex, 3-D arrangements. Transport of biological molecules through hydrogels can be accomplished by diffusion. However, it is heavily dependent on the porosity of the gel which can change depending upon desired substrate properties. To this end, a Bio Layer mask was designed to contain a series of holes to study their efficacy at enhancing nutrient and waste transport between the tubes interior and the surrounding media (Figure 14, B).

**Figure 11.** Image of fourteen Bio Layer sheets exhibiting short-edge rolling due to the presence of sticky strip on left side of Bio Layers.

**Figure 12.** The image to the left shows the edge of a silicon wafer coated with UL and imaged SL upon which Bio layers were patterned. The image on the right is the sticky layer mask. A correlation between self-assembly extent and speed bump density was observed.
Conclusion

The Bio Layers have demonstrated the ability to self-assemble into 3D tube-like structures. However, Bio Layer assembly in buffer solutions remains a challenge and requires further engineering of the Bio Layer films. The Sticky Layer has a practical role as an anchor and agent to control self-assembly direction. Rather than extending assembly time, it shows on/off characteristics. This was attributed to the Sticky Layer mask design which dictates speed bump size and density. Therefore, a new mask was designed to decrease both the size and density of the speed bumps while simultaneously broadening the range that these parameters can be analyzed. An MTT assay also showed favorable biocompatibility with mouse ESCs.
Future Directions

Re-designed Photomasks  The previous Sticky Layer photomask had a number of issues: hard to align, few regions for good comparisons, large speed bump size (≥ 625 μm²). Using L-Edit®, a new mask was designed (Figure 14, C) with the following properties: improved alignment marks, investigates speed bump homogenous density (total area coverage of 0%, 0.03%, 0.10%, 0.31%, 1.00% 3.12% and 10.0%) and investigates four speed bump sizes (25 μm², 49 μm², 100 μm² and 196 μm²). To compliment this redesign, two new Bio Layer masks were made to allow for easy swapping between two other BL masks designed for constant and variable BL aspect ratios (Figure 14, A, B).

Figure 14. Above are two BL designs and one SL design. The schematics above are a general layout and do not include labels printed on the masks. (A) Constant aspect ratio BL mask (width = 1500 nm, length = 3000 nm). (B) Non-constant aspect ratio mask (aspect ratio range from 1.0-4.0) with constant width (1500 nm). Smaller (4 x 4) regions contain aspect ratio = 2.0 contain holes differing in number and size. (C) Sticky Layer masks with sticky strips clearly visible. Larger strip regions contain decreasing speed bump density from left to right and four different speed bump sizes in each quadrant. Each Bio Layer mask is designed to be used with this Sticky Layer mask.

Hydrolyzing Sticky Layer  Current SL design relies on temporary adhesion to the scaffold to control self-assembly. It may be determined that this method is not adequate as a primary mechanism. One possible alternative is a chemical switch to release the scaffold through the introduction of lactone or anhydride monomers into the speed bump polymer. The hydrolysis of these monomers would make the speed bumps more similar to the “slippery” UL. A monomer could be chosen so that its rate constant
match with desired self-assembly times. Implementing this approach may require the separation of the SL between the sticky strip and the speed bumps.

**Biocompatibility** In order to fully integrate the Bio Roll-Up system into a useful scaffolding technology, more extensive biocompatibility studies must be done. The effect of peptide binding to Bio Layer 2 on cell adhesion and proliferation must also be studied. Finally, Bio Layer assembly must account for mechanical effects due to cell-laden Bio Layer 2. This can be studied using microspheres which already play an important role in biological fields.23

**Swelling Characterization** Procedures for measuring the swelling of these films must be put into place. Swelling ratios provide relevant information on the hydrogels properties and behavior. Parameters such as crosslink density can be calculated which will lead to better thermodynamic understanding of these polymers. Osmotic pressure can also be theoretically derived from Flory-Huggins theory.19 By knowing simultaneously knowing the molecular weight of the polymer chains, swelling ratio, elastic modulus and tube dimensions, a computer model could be created for a particular polymer system to assess how the scaffold could be rationally designed.

**Experimental Section**

**Synthesis of poly(HEMA-co-AA)** Hydroxyethyl methacrylate (47.105 g; 362 mmol), acrylic acid (2.9 g; 40 mmol) and azobisisobutyronitrile (AIBN) (1.98 g; 12 mmol) were dissolved in XX g (XX mL) of propylene glycol monomethyl ether (PM) and degassed with nitrogen for 5 minutes. The mixture was heated at reflux for 16 hours and then let cool to room temperature.

**Synthesis of poly(HEMA-co-MMA)** Hydroxyethyl methacrylate (9.0 g; 69 mmol), Methyl methacrylate (10.7 g; 107 mmol) and azobisisobutyronitrile (AIBN) (0.061 g; 0.37 mmol) were dissolved in 81.1 g (83.6 mL) of propylene glycol monomethyl ether acetate (PMA) and degassed with nitrogen for 20 minutes. The mixture was heated at reflux for 19 hours and then let cool to room temperature.

**Bio Layer 1 Formulation** Poly(HEMA-co-AA), ethoxylated glycerine triacrylate and Irgacure® 2959 were dissolved in PM at a total concentration of 20% w/w. The concentration between the dissolved
components were the following: poly(HEMA-co-AA) (80% w/w), ethoxylated glycerine triacrylate (10% w/w) and Irgacure® 2959 (10% w/w).

**Bio Layer 2 Formulation** Poly(HEMA-co-AA), ethoxylated glycerine triacrylate and Irgacure® 2959 were dissolved in PM at a total concentration of 20% w/w. The concentration between the dissolved components were the following: poly(HEMA-co-AA) (70% w/w), ethoxylated glycerine triacrylate (20% w/w) and Irgacure® 2959 (10% w/w).

**Sticky Layer Formulation** Poly(HEMA-co-MMA), tetramethoxymethyl glycoluril and Irgacure® CGI 1907 were dissolved in PM at a total concentration of 6% w/w. The concentration between the dissolved components were the following: poly(HEMA-co-MMA) (85% w/w), tetramethoxymethyl glycoluril (10% w/w) and Irgacure® CGI 1907 (5% w/w).

**Underlayer Formulation** Poly(HEMA-co-AA), tetramethoxymethyl glycoluril and K-PURE® TAG-2172 were dissolved in PM at a total concentration of 2% w/w. The concentration between the dissolved components were the following: poly(HEMA-co-AA) (65% w/w), tetramethoxymethyl glycoluril (25% w/w) and K-PURE® TAG-2172 (10% w/w).

**Stack Fabrication** The underlayer formulation is spin coated on a 100 mm silicon wafer at 2000 rpm for 45 seconds. The wafer is baked at 200°C for 120 seconds. The Sticky Layer is coated over the underlayer at 1000 rpm for 45 seconds. It is baked at 100°C for 90 seconds. The Sticky Layer is imaged with an exposure time of 10 seconds (~95 mJ/cm², i-line) followed by a post-exposure bake of 100°C for 120 seconds. It is then developed in 100% PM for 60 seconds. The Bio Layer imaging is done together to improve alignment. Bio layer 1 is spin coated at 1000 rpm for 30 seconds followed by a bake at 60°C for 120 seconds. Bio layer 2 follows the same procedure. Both Bio Layers are patterned using an exposure time of 300 seconds (~2.8 J/cm², i-line). The wafer is then developed in 20% PM solution in deionized (DI) water for 30 seconds. A rinse with DI water precedes a 20 second soak in 1x phosphate buffer saline (PBS). After the soak, the wafer is gently dried with air or nitrogen to leave behind the salts from the PBS. The wafer is then immersed in the “swelling” or “self-assembly” solution, DI water.

**MTT Assay** Samples were prepared by spin coating the experimental layers onto a 12 mm biocompatible glass slide. The positive control was an agar substrate. All samples were sterilized under UV light for 1 hour. Mouse epithelial stem cells were incubated in Gibco® media at 37°C for 48 hours. A 12 mM methylthiazol tetrazolium dye solution was prepared and added to each sample plate as well as a
pure medium control. The samples were incubated for 4 hours before the addition of SDS-HCl followed by an additional 4 hour incubation. The SDS-HCl solution was prepared by adding 10 mL of 0.1 HCl to a tube containing 1 g of SDS. Absorbance was taken at 570 nm.

References


