

NIH PROPOSAL

Multilayered Graphene Scaffold for Controlled Tissue Regeneration



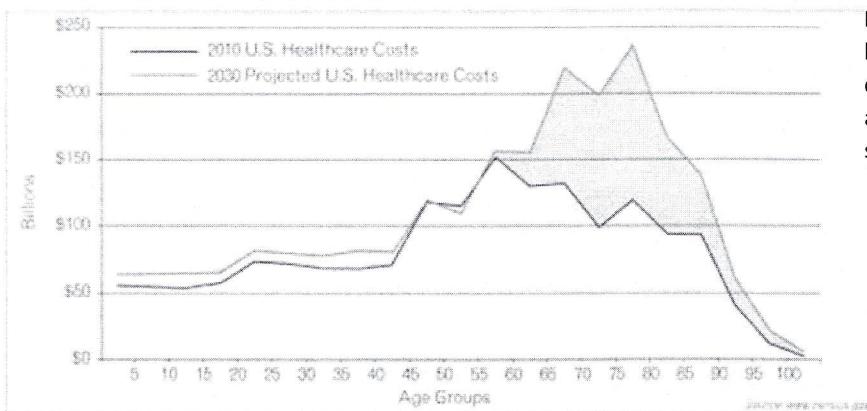
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Significance

Tissue regenerative therapy has the potential as a long-term solution to chronic diseases such as heart disease, cancer, stroke, pulmonary disease, diabetes, and osteoporosis. Current annual healthcare expenditures in the U.S. are approximately 2.5 trillion dollars, with 83 percent of healthcare spending associated with treating chronic diseases. Diseases such as these lead to a significant decrease in the patient's quality of life with very little hope of reducing such pain for the remainder of their life. It is projected that the number of chronic disease cases will increase over time as a result of the baby boomers, where patients over the age of 65 utilize more healthcare expenses for chronic diseases than all other age demographics. This will cause a significant increase healthcare costs and resources which might become a major deficit in our national economy in the near future (Staff, 2016).

The Cost Impact of an Aging Population



Projected healthcare costs. Comparison of healthcare costs between 2010 and estimated costs by 2030. Individuals over the age of 60 are more susceptible to healthcare costs as shown in the figure.

Figure 2. Projected healthcare costs. Reprinted from *Alliance for Regenerative Medicine*. Retrieved from <http://alliancerm.org/economics-rm>. 12/02/2016. Reprinted with permission.

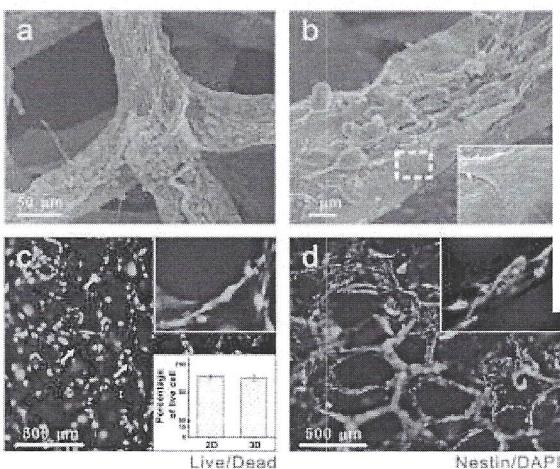
Tissue engineering therapy (TRT) is designed with the goal of improving clinical outcomes of chronic diseases, and reducing the cost of healthcare. By reducing the incidence of chronic disease cases, TRT could potentially save over 1.5 trillion dollars annually by reducing hospital care, physician clinical and professional services, pharmaceuticals, and nursing/home healthcare (Staff, 2016).

TRT has been vastly improved through the development of scaffolds. Scaffolds are devices designed to support, stimulate, and improve the healing of damaged tissue. In this way an individual is able to regrow or regain function of damaged tissue or organs with minimal scarring and acute/chronic pain. The use of graphene as a scaffolding material has generated great interest in research regarding its biocompatibility, cytotoxicity, and mechanical properties (Jayasuriya, Ashkan, & Jayatissa, 2014) (Li, et al., 2013, Vol 3) (Fan, et al., 2010, Vol 11). Graphene in a three-dimensional form has been considered for use in tissue engineering due to its biomimetic capabilities in resembling the extra-cellular matrix of functional tissue.

The gap in current research regarding 3D graphene foam (3D-GF) is understanding its biodegradation *in-vitro*. It is important that a scaffold is stable enough to allow cellular proliferation without being degraded prematurely. If degradation cannot be controlled *in-vitro*, then there is little confidence that 3D-GF can progress toward *in-vivo* testing. We will implement a novel approach for improving the stability of the 3D graphene by applying a coating composed of heparin-loaded graphene oxide (GO) or reduced graphene oxide (rGO). This research will commit to the NIH-mission to improve the design of medical therapies to be used for medical applications. This will result in improved quality of life of patients affected by chronic disease or injuries that require assisted healing of a damaged tissue. The outcome of our study will provide integral data on the use of 3D graphene as a functional scaffold to further progress toward an *in-vivo* model.

Innovation

Currently 3D-GF scaffolds utilize a nickel-based skeleton to support stem cell differentiation. The porous structure is advantageous for cell to cell signaling and guidance cues. This allows the cells to communicate with one another while regenerating damaged tissue. Studies have shown support of stem cell adhesion and induced differentiation toward a neural lineage when cultured on 3D-GF (Li, et al., 2013, Vol 3).



Cellular proliferation of stem cells on 3D-graphene foam scaffolds. Image a and b show SEM images of the foam under low and high magnification. White arrows in image c indicate cells that were not viable compared to cells that were viable. Image d, 5-day proliferation of neural stem cells on a 3D-GF scaffold *in-vitro*.

Figure 2. NCS adhesion and proliferation on 3D-GF Scaffold. Reprinted from "Three-dimensional graphene foam as a biocompatible and conductive scaffold for neural stem cells," by N, Li et al. 2013, *Scientific Reports*, Volume 3, page 3. Copyright 2013. Reprinted with permission.

The problem with current studies is that they lack complete degradation analysis on 3D-GF scaffolds *in-vitro*. Additional reports indicate that microorganisms could potentially degrade this scaffold before tissue has time to regenerate, but this has not yet been extensively studied (Akhavan, 2016, 4). The current coating technique used to layer graphene on graphene foam for biological studies is by way of Chemical Vapor Deposition (CVD). Studies have shown that this coating strategy is not preferred for coating graphene on 3D-GF due to its resulting hydrophobic surface chemistry. This could potentially reduce the biocompatibility of 3D-GF *in-vitro* (Akhavan, 2016, 4).

The proposed research is innovative because it will provide an in-depth analysis of the degradation of 3D-GF *in-vitro*. To fully understand the breakdown of graphene foam *in-vitro*, an additional coating layer will be applied to compare degradation rates. We will implement a dip-coating strategy to coat a stable film on 3D-GF. Experiments will determine stability of the GO and rGO coating on the 3D-GF scaffold. Studies have shown that heparin proteins can be functionalized to GO films to successfully coat titanium substrates (Chang-Jiang Pan, 2016, 63). Functionalizing heparin onto 3D-GF will bind pH-treated GO and rGO segments to the 3D substrate as a stable film (Da Yong Lee, 2011, 12). This process of applying a functionalized coating onto a graphene foam has yet to be tested with its use in tissue engineering.

A goal for using 3D-GF in tissue engineering is to create a functional scaffold that can be used in an *in-vivo* model. One of the most significant barriers preventing 3D-GF scaffolds from being tested *in-vivo*, is the lack of data on degradation *in-vitro*. A new horizon that our study will present, will be a complete *in-vitro* model encompassing a degradation analysis and coating strategy for commercialized 3D-GF scaffolds. This novel approach will provide new horizons for the scientific community to continue development of functional scaffolds for regenerative medicine and biomaterial development.

Approach

Aim 1: Confirm successful integration of graphene oxide layer on 3D graphene foam scaffolds. For this part of our experiment, the goal is to apply an outer coating to the 3D-graphene foam scaffold. The coating material we have chosen is GO or rGO. Graphene oxide has been shown to have excellent biomimetic capabilities due to the presence of functionalized oxygen groups. GO is a hydrophilic material, thus reducing the possibility of adverse cytotoxic effects. In order to apply this coating, we have chosen to use a dip-coating technique combined with protein anchoring to ensure that the 3D-GF scaffold coating is uniform and stable. The preceding citations and related discussion illustrate that current 3D-GF scaffolding models could potentially lack the stability needed to be used *in-vivo*. The porous structure of the scaffold allows for penetration by microorganisms. Furthermore, experiments suggest that the use of 3D-GF by itself shows brittleness and inflexibility which prevents its widespread use (Wang, et al., Vol 7, 2015). In order to effectively optimize the properties of three-dimensional graphene, we will provide an additional coating technique to reinforce the stability of current commercialized models. It is critically important to integrate a layer on the outside of 3D-GF which is the focus of this aim. Our approach requires that we confirm the successful integration of these graphene layers by analyzing the surface coating integrity of our scaffolds using Raman Spectroscopy, XPS, and SEM imaging.

Objective 1.1 Create GO and rGO material.

Experimental approach. Hummers method is a well-established method for chemical processing of graphite into GO and rGO as previously reported (Zhang, 2014). Graphite clumps will be added into a sodium nitrate/sulfuric acid solution until mixed homogenously. Potassium permanganate will then be added to oxidize the graphite into graphite oxide. To create rGO, GO will be further processed by mixing hydrazine hydrate with the GO solution. The final products will then be filtered and sonicated for 2 hours to create mono-layered rGO and GO.

Objective 1.2 Load heparin to graphene foam and functionalize to GO and rGO films.

Experimental approach. Previous literature presents a simple protocol which uses poly-dopamine (PDA) to coat heparin proteins onto graphene film (Chang-Jiang Pan a, 2016, vol 63) (Chong Cheng, 2013, vol 1). PDA will be adhered to both GO and rGO in aqueous solution through extensive mixing before pristine heparin is added to the solution. Graphene foam substrates will be pre-treated with dopamine hydrochloride in aqueous solution. Graphene foams will then be dip coated into heparin, and heparin loaded rGO/ GO solutions for 24 hours to ensure complete saturation of the different graphene coatings. Variables and controls for characterizing coating of rGO and GO films onto graphene foam through heparin loading will include the following: graphene foam without heparin coating, graphene foam with heparin coating only, graphene foam with heparin and GO coating, and graphene foam with heparin and rGO coating.

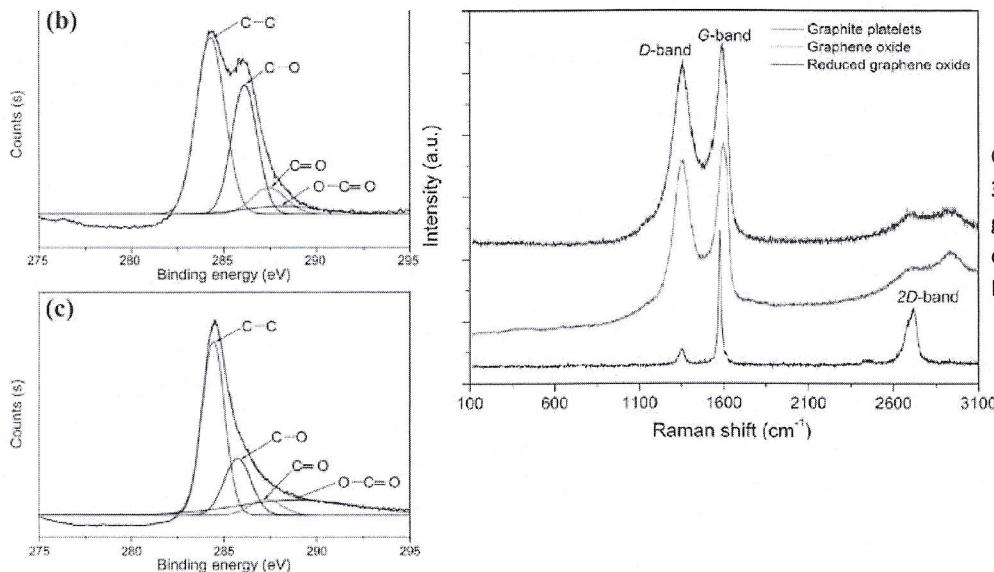
Objective 1.3 Characterize graphene coatings on Graphene foam.

Experimental approach. The coating iterations will be characterized using several methods. Surface chemical structures of each coating will be examined by X-ray Photoelectron Spectroscopy (XPS) to detect the presence of heparin loaded rGO and GO on graphene substrate. Further characterization by Raman spectroscopy will support XPS data by detecting heparin-loaded rGO and GO through Raman scattering of light. Topographical difference of the coatings will be determined using Scanning Electron Microscopy (SEM). Each analysis will be statistically evaluated with a sample size of 6 to detect significant differences in XPS and Raman spectroscopy data.

Expected Outcome: In this aim, we expect to produce rGO and GO iterations and functionalize them with heparin protein. Determination of successful coating on to 3D graphene foam is expected to be performed by SEM, X-ray Photoelectron Spectroscopy (XPS), and Raman spectroscopy. Since our aim is to effectively optimize the properties of three-dimensional graphene with an additional coating to reinforce stability, we expect to successfully integrate graphene oxide layers on to 3D-GF scaffolds by the end of this experiment. Our surface coating is expected to lack randomly distributed impurities and surface charges. Typical Raman spectra of high-quality GO and rGO will show similar graphical results to the figure below with a more define D-band due to increased amorphous carbon and oxidative

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groups on its surface. XPS graphical results will show increased C-O, C=O, and O-C=O binding energy peaks similar to figures (b) and (c).



Characterization of the coated 3D-GF scaffold. Figure (b) XPS graph of GO. Figure (c) XPS graph of rGO. Right figure. GO and rGO Raman spectra

Figure 3, Figure 5. Raman spectra and XPS graphs of GO and rGO. Reprinted from "Fabrication of Spirocyclic Phosphazene Epoxy-Based Nanocomposites with Graphene via Exfoliation of Graphite Platelets and Thermal Curing for Enhancement of Mechanical and Conductive Properties," by Feng et al. 2013, *I & EC Research*, Volume 52, page 10164. Copyright 2013 American Chemical Society. Reprinted with permission.

Aim 2: Provide analysis of degradation of graphene layers on the scaffold as a function of time under in-vitro conditions. The primary concern with current graphene-based models is their lack of degradation studies. In order to progress toward an *in-vivo* model, the integrity of these scaffolds must be studied under conditions that resemble the extracellular matrix of the cell. Our experiments intend to provide data on the degradation of current 3D-GF scaffolds, as well as ones that have been coated with graphene oxide and reduced graphene oxide. The scaffold without an additional coating will serve as the control. Scaffolds that are currently used in tissue engineering provide extensive studies on biodegradation rates, thus justifying the importance of providing such analysis. The preceding citations and related discussion illustrate that degradation is required for regeneration of natural tissue. Biodegradation employs a 4-step process that involves hydration, loss of tensile strength, loss of mass, and finally solubilization (Freed, et al., Vol 12 1994). Our experiments will mimic previous studies done on scaffolds that are being used commercially on our coated 3D-GF scaffold.

Objective 2.1 Degradation analysis of graphene foam coatings.

Experimental approach. Each graphene foam iteration will be suspended in Simulated Body Fluid (SBF) to closely mimic the ionic concentrations found in human plasma and placed in incubator. Scaffolds will be suspended for 6 weeks to allow for degradation of the coatings. Analysis of the degree of degradation will be performed at 1 week intervals until the end of the analysis. Degradation analysis will involve the following. Mass/ Weight ratios of each coating iteration to determine if mass has changed as a sign of degradation. Raman spectroscopy will determine if the surface coating is changing to reveal the heparin layer underneath as a sign of degradation. SEM will give a qualitative analysis of how the topography of the coated surfaces change with respect to time. SBF media, biomaterial production, and incubation parameters will be controlled during the degradation analysis to simplify statistical analysis. A sample size of 6 for each analysis will be sufficient to determine if significant difference in the data is present.

Expected outcome: In this aim, we are expected to provide in-depth analysis of the degradation of 3D-GF under *in-vitro* conditions. Degradation analysis, which is expected to be performed over a span of 6 weeks is expected to provide extensive data on degradation of 3D-GF *in-vitro* and provide insight to new ways in improving scaffold integrity to continue development of functionalized scaffolds. We expect to see a gradual reduction in the mass of coated 3D-GF

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scaffold. SEM images will show detailed changes in surface topography. We expect Raman spectra readings taken throughout the 6-week degradation analysis to show a decrease in D peaks due to the degradation of the graphene coating. The G peak will still be pronounced until the original 3D-GF scaffold is fully degraded.

Aim 3: Determine if the scaffold retains its biocompatible and antimicrobial properties *in-vitro* after coating. Once the scaffold has been coated, the surface chemistry will be analyzed to ensure that the scaffold maintains its biocompatibility. In addition, we will perform tests to determine if any bacterial contaminants are able to proliferate on the scaffold. The preceding citations and related discussion illustrate that graphene has excellent anti-microbial properties. This is one of the reasons it has generated interest in tissue regeneration. Studies have shown that 3D-GF scaffolds are biocompatible and prevent bacterial contaminants from growing on its surface (Li, et al., Vol 3 2013). In order to justify the use of a coating, we must show that the graphene does not have adverse cytotoxic effects. In addition, it must retain the antimicrobial benefits of graphene scaffolds currently being used. Previous experiments used Calcein-AM and EthD-I staining assay in order to evaluate the cytotoxicity of the 3D-GF. A TUNEL assay was used to determine if there were any abnormal cell apoptosis (Li, et al., Vol 3 2013). Our experiments will use similar assays to evaluate the cytotoxicity of our scaffold with a GO and rGO coating. Lastly, we will employ previous gram staining techniques used to evaluate the antimicrobial properties of the GO and rGO, which will comply with the focus of this aim. Experiments done previously reported that, “The random arrangement of GO layers in the porous foam architecture allowed it to exhibit excellent antibacterial activity...” (Jayanthi, et al., Vol 6, 2016). This data justifies the feasibility of antimicrobial studies, and further proves that 3D-GF scaffolds are excellent candidates in regenerative medicine.

Objective 3.1 Proliferation and cytotoxicity assay of graphene foam coatings.

Experimental approach. Each graphene foam iteration will be suspended in culture media with Rat- Adipose Derived Stem Cells (ADSC) in sterile 24-well flasks for 8 days. During culture, TUNEL assay and Calcein AM/ EthD-I assay will be performed on day 2, 4, 6, and 8 to determine if proliferation and cell death is present on the graphene foams. Biomaterial production, incubation parameters, cell passage, and culture media will be controlled during the culture analysis to simplify statistical analysis. A sample size of 6 for each analysis will be enough to determine if there is significant difference in the data presented. Our results will be compared to previously published literature on the biocompatibility of graphene foam without coating. (Li, et al., Vol 3 2013)

Objective 3.2 Gram staining for microbial activity on graphene foam coatings.

Experimental approach. Using both gram-negative and positive bacterial lines, we will swab the graphene foam coatings with the cell lines and allow the cultures to grow for 4 days. Gram staining will be performed at day 1, 2, and 4 for detection of both gram negative and positive bacterial culture growths. Each stain will be counted for number of bacteria in a given surface area of the graphene foam coatings. This will determine if bacterial growth is present on the coatings and determine if microbial or anti-microbial activity is present. Biomaterial production, incubation parameters, and culture media will be controlled during the culture analysis to simplify statistical analysis. A sample size of 6 will be sufficient to determine if significant difference in the data is present.

Expected Outcome: In this aim, we expect to develop a sophisticated biomaterial with advanced biocompatible and antimicrobial properties. We expect to determine the biocompatibility of the scaffolds by proliferation and viability assays of rat-derived stem cells on coated 3D-GF. We expect to determine the antimicrobial properties of the scaffolds by bacterial swabbing each layer *in-vitro*. This data will determine if 3D-GF altered by graphene coatings is able to retain the properties of current 3D-GFs. The figure below shows detection of cell viability on a graphene-based scaffold using a Calcein-AM stain. We expect to see similar results, showing increased cell viability over time on GO and rGO coatings.

Fluorescent image with Calcein-AM/ EthD-I stain. Results show cellular proliferation in the presence of graphene oxide for 7-day culture.

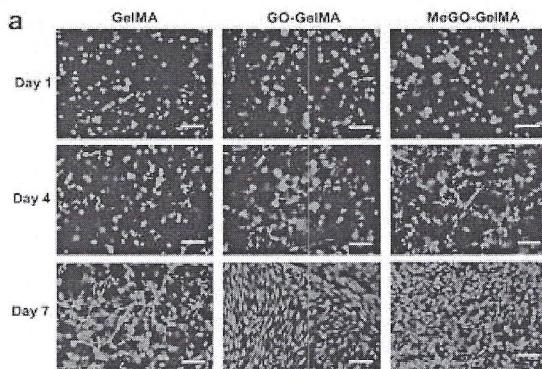


Figure 7. Calcein-AM/EthD-I stain. "Controlling Mechanical Properties of Cell-Laden Hydrogels by Covalent Incorporation of Graphene Oxide," by Cha et al. 2014, NIH Public Access, Volume 12, page 18. Copyright 2013. Reprinted with permission.

Potential Problems & Alternative Approaches

Our working hypothesis is that heparin loaded GO or rGO can be coated onto 3D graphene foam to provide stability and controlled degradation. Preliminary data from published works on graphene foam and protein loading strongly suggests that we can expect stable layering of GO and rGO on graphene foam by heparin protein anchoring. If instead, we find that GO/ rGO is not layered on graphene foam after dip coating we would interpret such a result to mean either incomplete saturation of heparin protein on the graphene foam structure or in-complete pretreatment of PDA on graphene foam structure. In this case, we would first employ alternative methodologies in self-assembling proteins onto graphene substrates to test for optimal coating parameters. Self-assembly parameters by dip coating can be altered to change the absorption rate of proteins on material surfaces by changing the following. concentration of proteins in solution, pH level of the mixing solution, and mixing appropriate catalysts and mixing procedures to improve reaction time. Using these methods for self-assembly we will troubleshoot the optimal material coating technique in the event that coating heparin loaded graphene on to graphene foam proves to be difficult.

Timetable

| | 1 Quarter | | 2 Quarter | | 3 Quarter | | 4 Quarter | |
|------------|-----------|---------|-----------|---------|-----------|---------|-----------|---------|
| | 2 month | 2 month |
| 4 Aim 1 | | | | | | | | |
| 5 Obj 1.1 | | | | | | | | |
| 6 Obj 1.2 | | | | | | | | |
| 7 Obj 1.3 | | | | | | | | |
| 8 Aim 2 | | | | | | | | |
| 9 Obj 2.1 | | | | | | | | |
| 10 Aim 3 | | | | | | | | |
| 11 Obj 3.1 | | | | | | | | |
| 12 Obj 3.2 | | | | | | | | |

Projected research completion time line. Red signifies specific aims for the project. Objectives for each aim are denoted in orange.

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