Photophysical and Chemical Processes Controlling the Emission Spectra of Rhodamine-Anthraquinone Sensors

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Motivation/Background: While a number of methods exist for the detection of biologically and environmentally significant cations, the use of fluorescent chemosensors is particularly beneficial due to their intrinsic sensitivity, selectivity, and practicality. The study of the mechanisms causing fluorescence changes in these sensors contributes to the understanding of how rhodamine- and anthraquinone-based sensors function. Additionally, the reduced rhodamine-anthraquinone sensor selectively fluoresces in the presence of Hg(II) cations.

Objective Statement: The goal of this research is to explain the spectral changes observed in an oxidized and a reduced rhodamine-anthraquinone sensor.

Method/Approach: The absorbance and emission spectra of the two rhodamine-anthraquinone sensors were determined before and after the addition of a series of metal cations. Fluorescence and absorbance competition studies were used to determine the selectivity of the sensors. Kinetic, titration, and solvent studies were also performed. Computational studies were used to investigate the structure of the sensors in the presence and absence of Hg(II) and Cu(II) as well as the contributions of energy transfer and electron transfer to the fluorescence quenching of the oxidized sensor.

Results: The fluorescence of the oxidized rhodamine-anthraquinone sensor is selectively enhanced in the presence of Hg(II) and Cu(II), and the emission of the reduced sensor is selectively enhanced in the presence of Hg(II). The fluorescence intensity of the oxidized sensor is smaller than that of the reduced sensor, which can principally be attributed to an electron transfer process.

Conclusions: The fluorescence of rhodamine-anthraquinone sensors is influenced by kinetics, the solvent concentration, the oxidation state of the ligand, and the relative concentrations of the ligand and the metal cations.

Validation of hits identified in an siRNA screen as positive regulators of ER stress-induced death.

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Motivation/Background: The Endoplasmic Reticulum (ER) plays a major role in the synthesis, folding and trafficking of secretory and membrane proteins. Physiological or pathological conditions can alter proper ER functions, which results in the accumulation of unfolded proteins in the ER and causes ER stress. Three ER-anchored receptors (termed IRE1, PERK and ATF6) sense the accumulation of unfolded proteins in the ER lumen, and respond by activating a set of signaling pathways known as the unfolded protein response (UPR) - aimed at resolving the stress. The UPR reduces the stress by promoting the expansion of the protein-folding apparatus, by reducing the synthesis of new proteins and by forcing degradation of the unfolded proteins (through ERAD and autophagy). However, when ER stress is too severe and/or prolonged, the UPR is insufficient to restore homeostasis, and turns into a toxic signal leading to cell death.

Objective Statement: So far, how UPR signaling switches from an adaptive to a pro-death response is mainly unknown. Similarly, the precise signaling pathways and molecular mechanisms regulating UPR-mediated cell death activation remain unclear. Gaining a better understanding on UPR regulation is therefore an important priority, especially knowing that ER stress-induced cellular dysfunction and death are associated with and contribute to many human diseases, including stroke, cardiovascular disease, diabetes, obesity, neurodegenerative diseases, inflammatory diseases, viral infections and cancer. All of these diseases are of growing prevalence in the Western world and thus very relevant to the future health of the European population. The laboratory has performed RNAi-based cellular screen to identify new proteins implicated in the regulation of UPR-mediated cell death. The research project will therefore consists in the in-depth study of one of the hits that emerged from these screens, using gain- as well as loss of function approaches. The aim is first to confirm the implication of the protein of interest in the studied cellular process and then to decipher the signaling pathways and molecular mechanisms regulated by this protein.

Method/Approach:

- Cell culture (primary cultures as well as cell lines)
- siRNA screen for Mouse Ubiquitin Conjugation library in MEF. (SMARTpool genetic screen)
- Modulation of protein expression in cells (Repression using siRNA)
- Signal transduction studies (Western blotting RT-qPCR)
- Cell death/survival studies (Cell Death quantification and DEVDase assays)

Results/Conclusion: Our obtained results do not suggest CUL7 is implicated in blocking tunicamycin induced cell death. However, further information about CUL7 protein expression is required to fully validate. In addition, our preliminary results show that RNF2 knockdown has no effect on blocking tunicamycin induced cell death either. Initial results for TRIM31 knockdown suggested potential blocking ability against tunicamycin induced cell death, which requires further validation. Moreover, our results indicated that capase-3 is required for caspase-8 activation during tunicamycin induced ER stress.

Novel 3D Electrospun Nanofibrous Scaffold for Bone Tissue Engineering

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Electrospun scaffolds closely mimic cell ECM nanofibrous structure and mechanical properties, making them ideal candidates for tissue engineering scaffolds, but current manufacturing methods limit electrospun polymer scaffolds to two dimensions, impeding clinical applicability. We introduce a novel manufacturing method that creates a three-dimensional (3D) electrospun scaffold for tissue engineering, with interconnected macroporous structure for increased effectiveness of cell viability, mechanical properties, and mimicking of natural cell ECM. Polycaprolactone (PCL) was chosen for its excellent biocompatibility, degradation rate in vivo and prior FDA approval for clinical applications. Manufacturing consisted of thermally induced nanofiber self-agglomeration (TISA) after electrospinning, followed by freeze drying of the agglomerates. Scaffold properties were characterized by SEM where fibers were determined to have diameter 200 nm-1 um with porosity averaging 96%. We hypothesize this scaffold design encourages cartilaginous differentiation of mesenchymal stem cells prior to osteogenic differentiation, more closely mimicking physiological endochondral bone formation than direct osteogenic differentiation. Biological experiments to study cell activity both in vitro and in vivo were performed using mouse bone marrow stromal cells (mBMSC), with a phase separation-porogen leached PCL scaffold (TIPS-P) used for comparison. In vitro gene expression was carried out through real-time PCR, and cell viability was observed with a live/dead cell assay kit. In vivo mouse model tests involved ectopic implantation of scaffolds, which were harvested and characterized through H&E and safranin-O stains. In vitro gene expression showed higher levels of osteogenic marker expression in the TIPS-P group, whereas the TISA group exhibited higher levels of calcium, chondrogenic gene expression, and percent live cells. Moreover, our in vivo data indicated BMP2/stem cells induced stronger endochondral bone formation after 4 weeks of transplantation. Collectively, the data suggest that our novel 3D electrospun nanofibrous TISA scaffolds are more effective carriers for stem cell-mediated functional endochondral bone formation by recapitulating natural bone development and repair.

Preparation, Characterization, In Vitro Drug Release, and Cellular Interactions of Tailored Paclitaxel Releasing Polyethylene Oxide Films for Drug-Coated Balloons

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Drug-coated balloons (DCBs) are used to treat various cardiovascular diseases. Currently available DCBs carry drug on the balloon surface either solely or in combination with excipients. Increasing evidence suggests that a significant amount of drug is lost in the blood stream during balloon tracking to deliver only a sub-therapeutic level of drug at the treatment site. Hence, there is a great need to develop a drug delivery carrier that can prevent the drug loss from the balloon surface during tracking, and then immediately deliver a therapeutic level of drug at the treatment site during the short balloon inflation time. This research is focused on developing paclitaxel (PAT) loaded polyethylene oxide (PEO) films (PAT-PEO) as a potential drug delivery carrier for DCBs. An array of PAT-PEO films were developed in this study to provide tailored release of >90% of drug only at specific time intervals, which is the time frame required for carrying out balloon-based therapy. The characterizations of PAT-PEO films using SEM, FTIR, and DSC showed that the films developed were homogenous and the PAT was molecularly dispersed in the PEO matrix. Mechanical tests showed that most PAT-PEO films developed were flexible and ductile, with yield and tensile strengths not affected after PAT incorporation. The SEM, FTIR, and phase contrast microscopy characterizations of films post drug-elution study showed that the dissolution of PEO under physiological conditions was the primary mechanism for PAT delivery. The viability, proliferation, morphology, and phenotype of smooth muscle cells (SMCs) interacted with control-PEO and PAT-PEO films were investigated. All the control-PEO and PAT-PEO films showed an excellent SMC growth inhibitory effect, with the degree of inhibition strongly dependent on the w/v% of the polymer used. Thus, this study demonstrated the potential for using PEO as an alternate system for delivering drug from the balloons.

Release of Nitric Oxide from Heparin Coated Cobalt-Chromium (Co-Cr) Surfaces

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Introduction: Drug eluting stents (DES) are currently used to treat coronary artery disease (CAD). The use of DES has highly reduced the occurrence of neointimal hyperplasia and thus prevented the renarrowing of the treated artery [1]. However, the anti-proliferative drugs such as paclitaxel, and sirolimus used in currently available DES have shown to impair vascular healing because of improper endothelialization and causing late stent thrombosis (LST) [2]. Also, some polymers used to obtain the sustained release of these drugs have shown to cause hypersensitive reactions thus leading to LST [3]. A stable heparin coating can reduce the chance of LST because of its excellent anti-thrombogenic property. Also, the delivery of nitric oxide (NO) has shown to inhibit the growth of smooth muscle cells (SMC) as well as promote endothelialization [4]. In this study, the stability of heparin coating on Co-Cr stent material using different concentrations of bovine serum albumin (BSA), pH of coating solution, and the incorporation of NO in the coating were investigated.

Methods: Co-Cr alloy plates (1 cm x 1 cm) were cleaned by sonicating in ethanol, acetone and methanol twice for 10 min each. A coating solution containing 1 % Heparin and 1 % BSA in deionized water was prepared at the pH of 11, 10, 9, 8, 7.4, 7, 6.5, 6, and 4 using either 1 M NaOH or 1 M HCl. A 100 µL of the obtained coating solution was microdrop deposited onto the Co-Cr alloy surface and the samples were dried in a vacuum oven for 20 h at 50 ˚C and -15 Hg vacuum. The stability of coating was determined by immersing each sample in 30 mL of PBS. After determining the pH to obtain better stability of coating (pH of 4), coating solutions containing 1 % heparin, and 1 % BSA at pH 4 were prepared with four different concentrations (0.01, 0.1, 0.5, and 1 %) of DETA NONOate incorporated. The samples were then immersed in PBS and placed in a water bath at 37 ˚C for 3 days. The amount of NO released into the PBS was determined by ozone-chemiluminescence process using Sievers nitric oxide analyzer (NOA). The morphology of coating was studied using scanning electron microscopy (SEM).

Results: The samples with pH of 6 to 11 showed poor stability in PBS. The coatings of these samples were dissolved in PBS within 2 min. The pH of 4 significantly improved the stability of coating and the coating was intact for upto 45 min which clearly showed that pH of the coating solution has an effect on the stability of heparin coating. The stability of NO in the PBS solution released from the coating was found to be good still after 3 days. SEM images showed that the coating became thicker with the increase in % of BSA in the coating solution. The images taken from the edge of the coating indicated that the coating was nicely adhered onto the Co-Cr alloy plates. The coating has become smoother with the addition of a plasticizer (glycerol) to the coating solution.

Conclusions: The stability of heparin coating was significantly increased by mixing it with BSA at pH of 4. NO was successfully released by incorporating DETA NONOate in this coating. A smooth coating was obtained by adding glycerol as a plasticizer.

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Title: PURIFICATION OF A NOVEL DRUG SUBSTANCE USING PREPARATIVE HPLC
Authors: LINDSEY L. MARSHALL; THERESE M. DOWNEY, PHD

Alumend has developed a novel naphthalimide chemistry that is capable of cross-linking biomolecules upon photo-activation to repair blood vessels in people who suffer from peripheral arterial disease (PAD). This new technology is called Natural Vascular Scaffold (NVS) system to reflect the ability of the drug substance to crosslink endogenous collagen imparting mechanical strength similar in nature to a native healthy artery. The NVS Solution is the drug product component of the NVS system. Initial purity analysis of the NVS drug substance post synthesis was determined to be 75% by HPLC. In preparation for entry into a Phase I clinical trial in the US, a preparative HPLC method was developed to purify the drug substance to greater than 99% purity.

Preparative HPLC is widely used in the pharmaceutical industry as a purification technique. Alumend has implemented this technique to further purify the NVS drug substance post synthesis. The development was based on the existing purity method used extensively with this product; however application of the chromatographic conditions were optimized with regulatory considerations in mind, specifically toxicity concerns from residual mobile phase solvents used during the preparative HPLC process. Only Class III (low toxicity) solvents, ethanol and acetic acid, were used as opposed to more traditional HPLC solvents. While solvent choice minimized regulatory concerns, the use of a highly viscous solvent posed development challenges. The optimization utilizing ethanol and acetic acid as elution solvents was performed on a semi-preparative scale and the flow rate, injection volume, amount loaded, column size, and particle size were evaluated. Once developed, the method was scaled by a factor of 17 for use with a preparative HPLC column and the fraction collection was automated using a Waters 2767 Sample Manager.

Fatty Acid Treated Electrospun Chitosan Nanofiber Membranes Reduce LPS-induced Macrophage Activation

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Motivation/Background: Orthopaedic and maxillofacial trauma is often accompanied by loss of large amounts of bone, and treatment is frequently inhibited by invasion of soft tissues. To overcome this, guided bone regeneration (GBR) membranes are used. Current materials used for these GBR membranes require a second surgical intervention for removal or, in the case of resorbable materials, have unpredictable degradation rates that impair their barrier function. Our lab has worked on electrospinning nanofiber chitosan membranes to address this issue. These membranes support wound healing and osteogenesis, while having controllable degradation rates. Their nanofibrous structure also increases surface area, making them suitable for drug delivery applications.

Objective Statement: The solvent used in the electrospinning process contaminates the membranes with acidic anions, which causes swelling of the material and loss of nanofibrous structure.

Method/Approach: Our lab proposed a novel approach to solving this problem via fatty acid (FA) treatments that increase hydrophobicity and protect the fiber nanostructure during the hydrolysis process to remove the acidic contaminants. In this study, the compatibility of FA treated membranes with cell cultured macrophages and the membrane's ability to inhibit nitric oxide production was tested. RAW 264.7 Macrophages were seeded on membranes in 4 groups: Na_2CO_3, Acetylated, Butyrylated, and Regenerated membranes (n=4). Cells were activated by exposure to 2µg/ml E. Coli LPS. Tissue culture plastic (TCP) and TCP+LPS were used as negative and positive controls, respectively. NO production was measured after 24 hours with Griess Assay. Cell TiterGlo Assay was used to quantify cells after 24 hours and NO production was normalized to cell number.

Results: NO production by macrophages was reduced by 15-25% by our FA treated membranes. Both Acetylated and Butyrylated membrane groups had statistically significant reductions when compared to TCP+LPS group (Figure 1). Macrophages presented growth significantly higher than controls on all membranes tested (Figure 2).

Conclusions: The FA treatments had no effect on *in vitro* compatibility with macrophages and reduced lipopolysaccharide-induced NO production when compared to TCP. These results suggest that the FA treated membranes may reduce the severity and/or length of the inflammatory process, contributing to improved wound healing and graft performance. Future work should be aimed at establishing cytocompatibility with fibroblast cell lines *in vitro*, loading and release characteristics using drug molecules of interest, and *in vivo* biocompatibility testing.

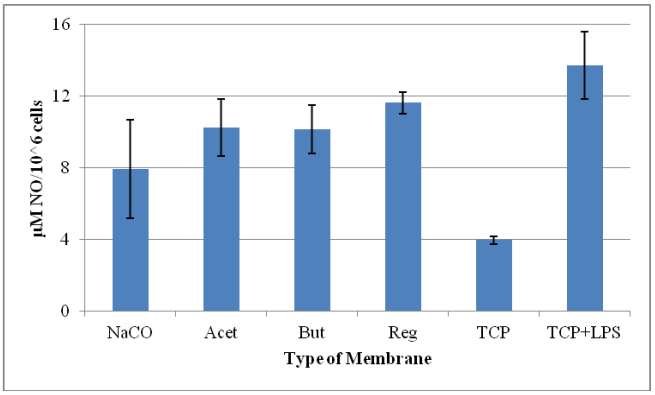

Figure 1. LPS-induced Macrophage Activation

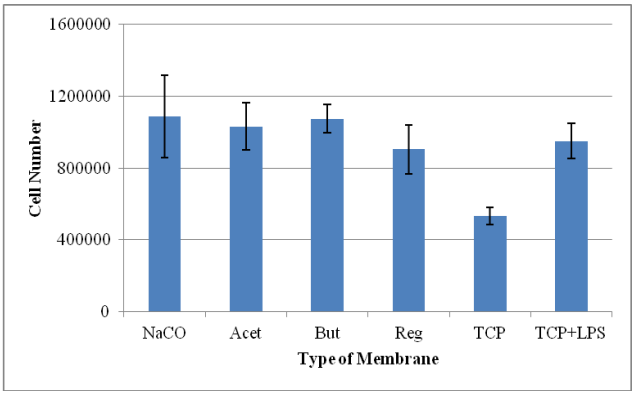

Figure 2. Macrophage Cell Number after 24 hr

Bioinspired Modulation of Stem Cells through Development of Biodegradable Porous Microspheres for Tissue Regeneration

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Abstract:

Injectable biomaterials are of great interest in regenerative medicine to facilitate stem cell-based wound healing, especially in the repair of irregularly shaped bone defects. Mesenchymal stem cells (MSCs), exhibit unique properties for tissue regeneration such as forming different tissues, promoting angiogenesis and exhibiting anti-inflammatory properties. Under appropriate conditions MSCs can differentiate into bone, cartilage and adipose tissue. Most current stem cell-based therapies use a 2D model, which can lead to premature differentiation and a loss of their multi-potential. 3D culture methods are emerging as a strategy to address these challenges. In this experiment we prepared 3D porous microspheres to act as stem cell carriers for bone and cartilage tissue regeneration. Poly (lactic-*co*-glycolic acid) (PLGA), a synthetic, biodegradable polymer, was used in this study to develop bioinspired microspheres. The microspheres were created with uniform size distribution of 100-250μm in diameter and average pore size of 20μm. We tailored the porosity and pore size by altering the concentration of porogen to yield uniform spheres. Previous studies suggest that scaffolds of similar micro and macro structure to our spheres are appropriate for cell growth *in vitro* and tissue formation *in vivo*. We seeded mouse MSCs onto spheres and used confocal microscopy to examine cell-microsphere interactions. Our hypothesis was confirmed as we observed cell expansion and migration toward the interior of the spheres. The data from this *in vitro* model demonstrated that our biomaterial supports cellular attachment and proliferation and is potentially a promising biomaterial strategy for stem cell-based tissue engineering and regenerative medicine.

Erythropoietin Immobilized Nano-fibrous Scaffolds for Tissue Regeneration

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Tissue engineering attempts to integrate biomaterials, cells, and drugs to offer an alternative to autologous bone grafts. Environment around bone defects is exacerbated by prominent inflammation and poor blood flow. In addition to erythropoietin's (EPO) traditional hematopoietic functions, EPO also has non-hematopoietic functions that assist tissue restoration and regeneration. Although the mechanism remains elusive, EPO can influence both inflammation and angiogenesis concurrently. However, high concentrations of EPO and systemic EPO delivery exert adverse effects such as thromboembolism and arterial hypertension. Aside from EPO, ARA290 peptide, a nonerythropoietic EPO derivative, was discovered and also has anti-inflammation ability.

In our lab, we aim to provide a localized and sustained drug delivery route to provide immuno-modulatory and angiogenic treatment simultaneously to promote bone and tissue regeneration. We hypothesize EPO/ARA290 can concomitantly exhibit pro-angiogenic and anti-inflammatory capability to promote endogenous tissue regeneration while reducing adverse effect. We will, for the first time, determine the function of ARA290 in bone regeneration. Finally, we will ascertain controlled, localized sustained delivery of EPO and ARA290 in *in vivo* model towards bone restoration.

In this study, bone marrow macrophages and macrophage cell lines were used and the cytokine expression was determined through ELISA assay. We will develop drug delivery process by incorporating EPO/ARA290 into nanospheres (NS). We also will immobilize PLGA NS onto biopolymer scaffolds. NS will be released from either synthetic or natural fibrous scaffolds; we will examine the drug release profile from the scaffolds.