

Design of the largest to-date scaffolded DNA origami from a 52K base pair single-stranded template

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Structural DNA nanotechnology, including scaffolded DNA origami, is rapidly developing as a versatile method for bottom-up fabrication of novel nanoscale materials and devices. However, the scale of these uniquely addressable structures is limited to the length of their single stranded scaffold, typically the 7249-nucleotide circular genomic DNA from the M13mp18 virus. We developed a scaffold using *E.coli* strain S3113, a lysogen of lambda wild-type phage, and a lambda/M13 hybrid virus to produce the desired 52K base pair DNA as a single-stranded virus. The sets of staple strands used to fold the larger scaffold were produced on a chip substrate surface. Additionally, predictions of the overall shape and mechanical properties of the structure are useful when developing effective designs at this scale. We designed distinct structures by over twisting and under twisting of the DNA helix, ultimately altering the intrinsic overall curvature of the structures. The computational resource CanDo predicted the expected 3D solution shapes, which was compared to our resulting structures as imaged by atomic force microscopy.

Laminin-Functionalized Biomaterials for Intervertebral Disc Regeneration

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Introduction: Laminins are heterotrimeric extracellular matrix proteins that mediate cellular functions, including cell adhesion and survival. Specific laminin isoforms and receptors have been identified in regions of the immature intervertebral disc (IVD), suggesting their involvement in regulating specific cell-laminin interactions in IVD growth and maintenance. The objective of this work is to develop a laminin functionalized biomaterial capable of supporting IVD cell adhesion, survival and maintenance of phenotype. Here, we examine the ability of PEGylated laminin-111 (LM111) to support nucleus pulposus (NP) cell adhesion. In addition, we evaluate the effect of PEG to LM111 ratio used in synthesizing PEG-LM111 conjugates on protein incorporation into three dimensional, photocrosslinkable PEG-diacrylate (PEG-DA) hydrogels.

Methods: Acrylate-PEG-N-hydroxysuccinimide (Ac-PEG-NHS, MW = 10kDa, Creative PEGworks) was added to a 2 mg/ml LM111 (Trevigen®) solution at a 10:1, 25:1, 100:1 or 500:1 molar ratio of Ac-PEG-NHS to LM111 to introduce functional acrylate groups for photocrosslinking. Wells of 96-well plates were each coated with one of these PEG-LM111 conjugates at 5, 10, or 25 µg/ml. Coated wells were then blocked with 3.75% BSA for 3 hours at 37°C to prevent non-specific adhesion. LM111 coated wells and BSA coated only wells were used as positive and negative controls, respectively. Porcine nucleus pulposus (NP) cells in serum free media were allowed to adhere to the LM111 and PEG-LM111 conjugate coated surfaces for 2 hours, and adherent cell numbers were determined via CellTiterGlo® assay.

Immunostaining of PEG-LM111 hydrogels was performed to assess the effect of PEG to LM111 ratio used in conjugate synthesis on the amount of LM111 incorporated into PEG-LM111 hydrogels. PEG-LM111 conjugates were mixed with photoinitiator (Irgacure 2959®, Ciba) and 10 kDa PEG-DA (Creative PEGworks) to a final concentration of 5 wt% PEG-DA, 0.1% Irgacure 2959®, and 200 µg/ml LM111 and polymerized upon exposure to UV light (365nm, ~3-4mW/cm²) for 5 minutes. Blank 5 wt% PEG-DA hydrogels and 5 wt% PEG-DA containing 200 µg/ml entrapped LM111 were photocrosslinked in the same manner for comparison. Frozen sections (20µm) of each construct were stained with a primary antibody specific to the γ chain of LM111 (Sigma L9393), followed by a goat anti-rabbit secondary antibody. Samples were imaged by confocal microscopy and acquired digital images were analyzed to determine the mean fluorescence intensity per image field as a measure of LM111 incorporated into each hydrogel.

Results: Results for NP cell adhesion to PEG-LM111 conjugates demonstrated decreased cell attachment with increasing ratios of PEG to LM111 used in conjugate synthesis, as compared NP cell attachment to unmodified LM111. Cell attachment was significantly reduced for conjugates synthesized with high ratios of PEG to LM111 (100:1 or 500:1); however, cell attachment numbers for PEG-LM111 synthesized with a 25:1 ratio of PEG:LM111 were greater than 89% of values for NP cell attachment to native LM111.

Immunostaining results demonstrate that the ratio of PEG to LM111 used in PEG-LM111 conjugate synthesis affects the amount of LM111 incorporated into the hydrogel, as mean fluorescence intensity per image field increased with increased PEG to LM111 ratio. Fluorescence images show more intense staining for LM111 in hydrogels containing PEG-LM111 conjugate synthesized with a high PEG to LM111 ratio (500:1) as compared to those containing PEG-LM111 conjugates synthesized with a low ratio of PEG to LM111 (25:1) or physically entrapped LM111.

Discussion: These results demonstrate that PEG-LM111 conjugates synthesized using a PEG to LM111 ratio of 25:1 maintain their ability to support NP cell attachment in a manner similar to unmodified LM111. At this ratio, immunostaining results suggest that LM111 incorporation into photocrosslinked PEG-DA hydrogels is increased compared to PEG-DA gels containing

physically entrapped LM111. Together, these findings demonstrate that the addition of functional acrylate groups onto native LM111 through the use of a heterobifunctional Ac-PEG-NHS allows for increased protein incorporation into PEG-DA hydrogels without significantly altering the proteins ability to mediate cellular function. Therefore, LM111 functionalized PEG hydrogels may be useful for promoting IVD cell attachment to matrix via specific mechanisms while promoting the rounded cell morphology and unique phenotype of IVD cells.

Infusions of umbilical cord blood-derived endothelial cells equal or surpass human aortic endothelial cells at treating endothelial injury in vein grafts.

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Objective: To assess the hypothesis that infusions of human umbilical cord blood derived endothelial cells (HCBECs) perform as well as adult derived human aortic endothelial cells (HAECs) in preventing thrombosis, promoting endothelium healing, and preserving adaptive neointimal thickening of vein grafts.

Methods and Results: HCBECs or HAECs were infused into carotid interposition vein grafts in NOD.CB17-Prkdc^{scid}/J mice. The vein-grafts were harvested postoperatively at four weeks to examine intimal thickening, two weeks to examine coverage of vein grafts, and at approximately one week to examine Evans Blue dye uptake. Unlike untreated vein grafts, all grafts receiving ECs were patent without any occlusion. Four week after formation of the vein grafts, intimal thickening was similar for grafts receiving 2 million HCBECs or an identical number of HAECs. Two week samples received 1 million cells, and HCBECs covered approximately 20% more luminal perimeter than HAECs ($P < 0.05$). For studies of Evans Blue staining, animals received 0.5 million cells. Animals receiving HCBECs exhibited less dye uptake in vein grafts than did animals receiving HAECs ($P < 0.05$), suggesting that the HCBECs promote more rapid endothelial healing than did HAECs. In vitro dynamic adhesion experiments performed at 0.5 dyne/cm^2 showed that HCBECs had equivalent adhesion to HAECs *in-vitro*. In contrast, HCBECs demonstrated greater migration towards Iloprost than did HAECs ($P < 0.05$), suggesting some difference in paracrine mediated responses of HCBECs and HAECs.

Conclusions: Infusions of HCBECs are as efficacious as HAECs at preventing thrombosis and preserving adaptive neointimal thickening of vein grafts in a mouse model. Moreover, HCBECs are more effective than HAECs in limiting changes to endothelial permeability shortly after establishment of the vein graft. Infusions of HCBECs present a promising, practical therapeutic option for reducing the large number of acute vein graft failures occurring yearly.

Genetic reprogramming at the single-cell level

Tyler Gibson, Charles A. Gersbach

The emerging field of regenerative medicine offers many promising approaches to heal tissues that are damaged or diseased. Regenerative therapies often involve a cell-based approach in which cells are coaxed into desired phenotypes. Genetic reprogramming uses gene regulatory factors to alter cell lineage commitment, offering a powerful tool to direct cellular differentiation.

The development of genetic reprogramming as a reliable tool for therapeutic use requires tight control of cell behavior. However, recent advances in the quantitative measurement of gene expression in single cells have documented a remarkable degree of variation between cells. This heterogeneity in cell behavior is obscured in measurements of the bulk behavior of cell populations. We differentiated a monoclonal line of immortalized myoblasts and stained for markers of differentiation and demonstrated that cellular differentiation states are different between cells, with only a fraction of cells transitioning to a differentiated phenotype.

Our objective is to study genetic reprogramming at the single-cell level using a genomic red fluorescent reporter of cell differentiation. We have developed a lentiviral system that allows for tunable and quantifiable expression of the transcription factors Runx2 and MyoD that induce cells towards bone and muscle lineages, respectively. We have demonstrated high transgene expression levels when fully activated and a dose-dependent response to the inducer concentration. Additionally, we have verified the activity of the delivered reprogramming factors in bulk population assays including qRT-PCR and Western blotting for markers of differentiation.

Ongoing work involves the production of cell lines that contain stably integrated reporter genes that activate GFP in response to bone or muscle differentiation. The simultaneous fluorescent measurement of both the level of reprogramming factor expression and transactivation of target promoters in live cells will enable us to monitor the cells' behavior during reprogramming. This information can lead to better control over cell-based therapies when applied in a regenerative context.

Characterization of a Self-healing Poly(methyl methacrylate) Bone Cement Containing Encapsulated Tissue Adhesive

Alice B.W. Brochu, William J. Chyan, Gregory A. Evans, and William M. Reichert

Self-healing materials are a rapidly emerging class of composites with applications intended for use in the civil, mechanical, electrical, and aerospace industries. These materials hold the potential for significantly extending functional lifetimes by repairing and preventing microdamage. The encapsulation of a biocompatible water-reactive tissue adhesive, 2-octylcyanoacrylate (OCA), used as a substitute for sutures in surgeries was examined to assess the practicality of designing a self-healing poly(methyl methacrylate) (PMMA) bone cement. Interfacial polymerization of a polyurethane prepolymer with 1,4-butanediol was used to encapsulate OCA utilizing an oil-in-water emulsion.

Scanning electron microscopy (SEM) was used to study capsule morphology, size, and shell thickness at various agitation rates. Thermogravimetric analysis (TGA) was used to determine the percent fill and shelf life of the capsules. ASTM F451, ASTM D638, and ISO 5833 were followed to assess the compressive, tensile, and bending properties of commercial PMMA matrices containing varying amounts of OCA-containing capsules.

Increasing the agitation rate from 350 to 1100 rpm during capsule fabrication decreased the average capsule diameter from $222 \pm 56 \mu\text{m}$ to $74 \pm 19 \mu\text{m}$, and decreased the average shell thickness from $6.3 \pm 2.6 \mu\text{m}$ to $1.6 \pm 0.5 \mu\text{m}$ (average ± 1 standard deviation). The OCA content in the core decreased from 58% in capsules made at 350 rpm to 46% in capsules made at 1100 rpm while shell content increased correspondingly from 37% to 47%. (Note: the presence of oil was consistently less than 7% in capsules made at all agitation rates.) A 4.9% reduction in the core content of the capsules was observed over a 14 day storage period with a total of 6.6% reduction after 56 days. Capsules were successfully incorporated into Palacos R bone cement matrices, surviving the mixing and heat associated with matrix curing. Compression testing following ASTM F451 revealed that the maximum strength decreased only 6.8% with incorporation of 10 wt% capsules but that above 10 wt% weakened the matrix below acceptable limits. Above 25 wt%, the compressive strength steadily decreased with a reduction of 41% seen in samples made with 40 wt% capsules. The Young's modulus behaved in a similar manner, decreasing with increasing capsule content; however, at 10 wt%, the highest allowable capsule content to maintain the compressive strength, the Young's modulus remained unchanged from a pure PMMA matrix. Preliminary tensile results indicate the tensile strength is only 70.2% of the compressive strength in specimens containing no capsules. Experiments are currently underway to determine the effects of capsule content on the tensile strength of the matrix; inclusion of 10 wt% capsules have been found to reduce the ultimate tensile strength by nearly 60%.

OCA-containing microcapsules possessing regular, spherical morphology were created via interfacial polymerization of a polyurethane prepolymer with a small chain diol. Core content comprised more than half of the microcapsule volume at all agitation rates with little weight loss observed after 8 weeks of storage. Future work will continue investigating the effects of the self-healing system on the bulk PMMA tensile, bending, and fatigue properties, assessing the healing efficiency of the incorporated system via crack propagation analyses, and examining the response of MG63 human osteosarcoma cells to the self-healing PMMA formulation.

***In situ* Grafting of a PEG-based Polymer Brush from the C Terminus of GFP via Sortase-mediated Initiator Attachment**

Stacey Qi

Covalent conjugation with stealth polymers has been a commonly used method to improve the delivery outcomes of therapeutic proteins and peptides. Conventional “grafting to” techniques typically result in low yield and difficulty in product separation. Even with the recent advent of several “grafting from” techniques, conjugation is still mostly achieved by exploiting promiscuously distributed reactive site chains, yielding large variability in the final product and significantly compromised bioactivity. To address these limitations, we demonstrate the use of sortase A (SrtA) from *Staphylococcus Aureus* to site-specifically attach an initiator solely at the C-terminus of Green Fluorescent Protein (GFP), followed by *in situ* growth of a stealth polymer brush, poly(oligo(ethylene glycol) methyl ether methacrylate) [poly(OEGMA)] by Atom Transfer Radical Polymerization (ATRP). The enzymatic sortase reaction is performed in mild physiological conditions with high specificity of action and near-complete product conversion. Subsequent *in situ* ATRP yielded stoichiometric conjugates with low polydispersity, high yield, and uncompromised bioactivity. This approach marks a useful new addition to the toolset of protein-polymer conjugation technology.

Visualizing lipid reactivity of HIV-1 antigen and neutralizing antibodies using atomic force microscopy

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Evidence suggests that lipid membrane interactions with rare, broadly neutralizing antibodies (NAbs), 2F5 and 4E10, play a critical role in HIV-1 neutralization. The objective of this research is to understand how lipid membrane properties, such as lipid domain organization contribute to 2F5/4E10 membrane interactions and antigen localization at the membrane interface, with the ultimate vision of guiding immunogen designs. Recent immunization studies have shown that induction of antibodies that avidly bind the gp41-MPER antigen is not sufficient for neutralization. Rather, it is required that antigen designs induce polyreactive antibodies that recognize MPER antigens as well as the viral lipid membrane. However, the mechanistic details of how membrane properties influence NAb-lipid and NAb-antigen interactions remain unknown. Furthermore, it is well established that the native viral membrane is heterogeneous, representing a mosaic of lipid rafts and protein clustering. However, the size, physical properties, and dynamics of these regions are poorly characterized and their potential roles in HIV-1 neutralization are also unknown.

To understand how membrane properties contribute to 2F5/4E10 membrane interactions, we have engineered biomimetic supported lipid bilayers (SLBs) and are able to use atomic force microscopy to visualize membrane domains, antigen clustering, and antibody-membrane interactions.

Our results showed that lipid domains were easily observed for simple binary membrane constructs and for complex, biomimetic HIV-1 model membranes. Localized binding of HIV-1 antigens (MPER₆₅₆) and NAbs were observed to interact preferentially with the most fluid membrane domain. This supports the theory that NAbs may interact with regions of low lateral lipid forces that allow antibody insertion into the bilayer. NAbs were also observed to cluster at the edge of certain domain interfaces suggesting NAbs affinity for high interfacial energy regions of the lipid membrane.

Comparison of Mixed and Lamellar Co-Culture Spatial Arrangements for Tissue Engineering Capillary Networks in vitro

Erica Brown Peters, George A. Truskey

Co-culture of endothelial cells and smooth muscle cells in vitro represent a promising method for tissue engineering capillary networks in vitro. In this study we examined whether the spatial arrangement of direct co-cultures affected endothelial cell migration, network morphology, and angiogenic protein secretion. Human umbilical cord blood derived endothelial cells (hCB-ECs) were grown in co-culture with human aortic smooth muscle cells (SMCs) in either a mixed or lamellar spatial arrangement and analyzed over a culture period of twelve days. Cell migration results indicate lower persistence times, higher speeds, and lower random motility coefficients in mixed hCB-ECs versus lamellar hCB-ECs during the first 24 hours of culture. By day twelve of co-culture, mixed systems demonstrated greater anastomoses and capillary loop formation than lamellar systems as evidenced by a higher number of branch points, angle of curvature between branch points, and percentage of imaged areas covered by networks. The network morphology was more uniform in the mixed systems than the lamellar systems with fewer endothelial cell clusters present after several days in culture. Proteome assay results indicate down regulation of pro-angiogenic proteins IGFBP-2, IL-8, and MCP-1 in lamellar systems compared to mixed systems. These results indicate that the spatial arrangement of ECs and SMCs in co-culture affects microvessel formation in vitro with mixed systems more conducive for microvessel formation than lamellar systems.

Genetic correction of dystrophin by engineered nucleases

David G. Ousterout, Pablo Perez-Pinera, Matthew T. Brown, Charles A. Gersbach

Duchenne Muscular Dystrophy (DMD) is the most common hereditary monogenic disease, occurring in about 1 in 3500 male births. DMD is caused by a genetic defect in dystrophin, an essential musculoskeletal protein. The absence of dystrophin leads to muscle weakness and wasting, resulting in fatal respiratory and cardiac disease. At present, there are no treatments that can effectively address the poor life expectancy and quality of life of these patients. Recent studies have demonstrated the potential of a new class therapeutics based on targeted gene editing by designer nucleases. These nucleases take advantage of natural DNA repair mechanisms to create desired changes to a genetic sequence. Importantly, zinc-finger nucleases (ZFNs) against the HIV-1 co-receptor CCR5 are in an ongoing Phase 1/2 and two Phase 1 clinical trials, demonstrating the feasibility of this type of therapeutic approach. Other studies are utilizing ZFNs to correct genetic mutations associated with sickle cell anemia, X-linked severe combined immunodeficiency, hemophilia B, and alpha-1 anti-trypsin deficiency.

This project utilizes synthetic nucleases to edit and correct the dystrophin gene as a novel potential therapy for DMD. The advantage of this method is that the native dystrophin gene is restored, presumably including all of the major isoforms and functions of dystrophin. We designed ZFNs and TALE nucleases (TALENs) targeted to exon 51 of the human dystrophin gene that potentially address greater than 13% of all DMD mutations. ZFNs were generated using modular assembly (MA) and Context-Dependent Assembly (CoDA). TALENs were generated using the Golden Gate Assembly method. Five MA-ZFNs, seven CoDA ZFNs, and twelve TALENs were tested for gene editing activity at the endogenous dystrophin gene in human K562 cells. Two MA-ZFNs, three CoDA ZFNs, and four TALE nucleases were highly active, modifying 6-13% of alleles as measured by the Surveyor nuclease assay. One ZFN of interest binds directly in exon 51 and mediated homology-directed repair in 6% of alleles using plasmid- and single-strand oligonucleotide-based donor templates. One TALEN mediated high-efficiency gene editing in 13% of alleles. Assuming that one third of these NHEJ events results in reading frame restoration, this approach has a potential gene correction rate of up to 4.3%. These nucleases were also active in immortalized myoblasts from DMD patients with an NHEJ rate of 1-12% as measured by Surveyor. We also detected successful homology-directed repair in these DMD cells after co-delivering the designer nucleases with a donor template designed to correct the dystrophin gene. We predict that this will lead to restored dystrophin function and expression in the corrected cells.

Ongoing studies are assessing dystrophin gene correction and protein restoration in DMD patient-derived myoblasts in vitro and by in vivo muscle transplantation into immunodeficient mice. The development of these nucleases therefore presents a transformative approach to treating DMD.

Light-inducible spatiotemporal gene regulation using customizable engineered transcription factors

Lauren Polstein, Charles A. Gersbach

The growing complexity of scientific research and gene-based medicine demands further development of gene regulation systems. The ability to safely and easily control gene expression in a spatial, temporal, tunable, and reversible manner is critical to many potential applications in gene therapy, regenerative medicine, tissue engineering, metabolic engineering, synthetic biology, and basic genetic research. To develop an approach that meets these criteria, we engineered a novel gene regulation system consisting of a combination of light-sensitive proteins and programmable zinc-finger transcription factors to enable reversible, repeatable, and spatially patterned transcriptional activation. This system, Light Inducible Transcription with Engineered Zinc finger proteins (LITEZ), takes advantage of two light-inducible dimerizing proteins found in *Arabidopsis thaliana*: GIGANTEA (GI) and the Light Oxygen Voltage (LOV) domain of FKF1. The chimeric proteins GI-ZFP (GI fused to a Zinc Finger Protein) and LOV-VP16 (LOV fused to the VP16 transcriptional activation domain) function as a molecular switch that initiates transcription when activated by blue light. A significant advantage of this system is that the zinc finger domain can be customized to bind nearly any target sequence, thus theoretically allowing spatiotemporal control of the expression of any gene. We used GI-ZFPs that target three different sequences ranging from 12 to 18 base pairs, as well as reporters for each GI-ZFP containing 3 to 9 copies of the respective GI-ZFP binding site upstream of a gene encoding luciferase or green fluorescent protein (GFP). We transiently transfected HeLa, HEK 293T, or MCF-7 cells with the luciferase reporter vector and LOV-VP16 alone or GI-ZFP alone and showed that illumination with blue light did not increase expression of luciferase. However, when LOV-VP16 and GI-ZFP were expressed together, exposure of cells to 24 hours of pulsing blue light resulted in a 53-fold increase in luciferase expression compared to transfected cells that were not illuminated with blue light. This light-inducible activation of gene expression is repeatable and reversible: luciferase expression increased when cells were exposed to blue light and decreased upon removal of blue light for two cycles. Furthermore, luciferase expression was tunable over a wide range by varying the number of ZFP binding sites in the transgene. Cells transfected with a luciferase reporter containing 3, 6, 7, or 9 binding sites exhibited a 4-, 22-, 38-, or 53-fold increase in luciferase expression, respectively, compared to non-illuminated transfected cells. Additionally, patterned illumination of HEK 293T cells transfected with a GI-ZFP, LOV-VP16, and a reporter vector containing 9 copies of the ZFP binding site upstream of GFP resulted in a corresponding spatial pattern of GFP-expressing cells. Patterned features as small as 500 micrometers in width were easily achievable.

In summary, LITEZ is a novel light-sensitive molecular switch that regulates a transgene in a specific, reversible, tunable, and spatiotemporal manner. Given the increasing interest in tissue engineering of multicellular constructs requiring complex spatiotemporal gene expression patterns, we expect there will be several promising applications for this system.

Formation of Substrate with Multiple Protein Gradients for Cell Migration Studies

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Statement of Purpose: Biomolecular gradients have been revealed to play roles in a wide range of biological phenomena. These include the immune reaction, development, inflammation, wound healing, and cancer metastasis. Characterization of these processes requires the capability to precisely spatially control the environment that the cells are exposed to. Cell signaling and interactions can be best studied when exposed to biomolecule gradients that are quantifiable, controllable, and mimic those that are present *in vivo*.

There have been many methods to create surface gradients [1][2][3], however most of these gradients only consist of one signaling molecule. An understanding of the mechanisms of many biological phenomena requires investigation of cell behavior due to multiple overlapping biomolecular gradients. Our project goal is to create multiple, well defined, quantifiable gradients of biomolecules to study cell signaling and cell migration. We are hoping to fabricate a uniform surface that can be spatially photoactivated, using the photolabile molecule benzophenone, forming multiple gradients of macromolecules.

Methods: Quartz slides were silanized, resulting in covalent linking of thiol-terminated groups to the surface. Benzophenone-4-maleimide was then covalently attached to the reactive thiol groups creating a uniform substrate of benzophenone. Benzophenone (BZP), a photoactivatable crosslinking molecule, can be activated by UV light.

UV light can be modulated to spatially alter the benzophenone activation and protein binding. This results in a gradient of this benzophenone activation molecule. Once activated, the benzophenone covalently binds methyl groups of surrounding molecules, method adapted from [4]. Signaling molecules will be introduced to this system thus creating a gradient of the macromolecules. Benzophenone moieties that do not form covalent bonds with the first molecule can be reactivated with UV light. As a result of this multiple-activation characteristic, it is possible to create more than one gradient of different molecules on the same substrate. Contact angles were measured and x-ray photoelectron spectroscopy (XPS) was used to verify the presence of uniform monolayer of thiol and benzophenone. Fluorescent protein will be used to verify the presence of the gradient. The fluorescent protein will be visualized by using a fluorescent scanner.

Results: First to ensure that the thiol chemistry was in fact decorating the surface of the quartz slide, XPS results were evaluated. The XPS data confirmed that there was benzophenone on the slides. As shown by the increase in area under the nitrogen curve, benzophenone is present on the experimental slide as compared to the thiol control slide. The increase in nitrogen demonstrates the maleimide group linking the photoactivatable benzophenone to the thiol group. The increase in nitrogen indicates benzophenone functionalization. After verifying the benzophenone chemistry on the quartz slides, a fluorescent dummy protein can be used to prove the presence of the gradient.

Conclusions: Preliminary data verifies that we have a photoactivatable surface. We are hoping to fabricate a uniform surface that can be spatially photoactivated forming multiple gradients of macromolecules.

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Design principles for thermosensitive nanoparticles

Jonathan McDaniel, Jayanta Bhattacharyya, Chris Radford, Ashutosh Chilkoti

Chimeric polypeptides (CPs) are a class of recombinant biopolymer derived from elastin-like polypeptides (ELPs) that combine an environmentally sensitive conjugation domain with the ability to rapidly shift from a soluble state to an insoluble coacervate in response to a specific temperature trigger provided by the parent ELP. We have previously shown that attaching multiple copies of doxorubicin to the chain end of the CP results in the assembly of monodisperse nanoparticles that display excellent pharmacokinetics and antitumor efficacy in murine cancer models. In an effort to fully understand the parameters controlling CP nanoparticle assembly, we have characterized a series of eight CP libraries of varying compositions and chain lengths as well as a series of small molecules used to drive assembly. We have found that CP assembly can be initiated by the conjugation of hydrophobic molecules or hydrophobic peptides to the chain end of the ELP, provided that the distribution coefficient (LogD) of the attached moiety is greater than a threshold value. The architecture of the nanoparticle (hydrodynamic radius, number of chains per nanoparticle) and the transition temperature are functions of both the ELP composition and the chain length. In contrast to ELP-conjugates (unassembled unimers), these CP nanoparticles display a thermal response that is near-independent of polymer concentration, number of attached molecules, and the molecule hydrophobicity. The application of these findings toward developing thermally sensitive therapeutics is ongoing.

Digital switching of cell-penetrating peptide density for controlled cellular uptake

Sarah MacEwan, Ashutosh Chilkoti

Cell-penetrating peptides (CPPs) are a class of materials valued for their ubiquitous cellular internalization in a variety of cell types. Due to their non-specific mechanism of uptake, CPPs must be carefully controlled to achieve targeted delivery of associated cargo. Current strategies to activate CPP function rely on intrinsic triggers at the targeted disease site, and are thus limited by the heterogeneity of these disease characteristics. We present an alternative strategy for controlling CPP function, in which an extrinsic activation trigger circumvents the heterogeneity of disease phenotypes. In our approach CPP function is controlled by modulating their local density with the temperature-controlled self-assembly of elastin-like polypeptide nanoparticles. We anticipate that this thermally triggered CPP activation will allow targeted delivery of CPP-associated cargo to a variety of disease targets.

Understanding the effects of early stage tissue response on the performance of implantable glucose sensors

Matthew T. Novak, Fan Yuan, William M. Reichert

Contemporary implantable glucose sensing paradigms are only approved by the FDA for up to one week. Recent research has suggested that interaction between the implanted tissue and the sensor is the main culprit in short sensor life. Early interactions between the sensor and the tissue are mediated by two processes: protein adsorption and cellular adhesion. To assess how these affect sensor performance, glucose sensors were immersed in both whole blood and blood constituents *in vitro*. Whole blood attenuated sensor signals relative to platelet poor plasma (PPP), suggesting that protein adsorption has little effect on sensor attenuation. Furthermore, the presence of cells proximal to the surface was found to be responsible for the decrease in sensor signal. These findings were supported by computational modeling and suggest that decreasing sensor function is a result of the environment around the sensor creating a deleterious environment for sensing.

Adhesive Properties of Late Outgrowth Peripheral Blood Endothelial Progenitor Cells

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Late-outgrowth peripheral blood-derived endothelial progenitor cells (PB-EPCs) may be obtained from patients non-invasively and cultured *in vitro* to isolate a cell population with endothelial cell surface markers and properties. However, characterizing the adhesive properties of PB-EPCs is critical for their acceptance as an autologous cell source for endothelialization of tissue engineered vascular grafts (TEVGs). Preliminary data suggests that PB-EPCs isolated from coronary artery disease (CAD) patients exhibit similar adhesive properties as human aortic endothelial cells (HAECs). Short-term spreading rate experiments over fibronectin (FN)-coated glass or a confluent layer of smooth muscle cells (SMCs) indicated that PB-EPCs spread to a larger average area than HAECs on FN-coated surfaces and SMCs, and PB-EPCs and HAECs aligned along the long axis of underlying SMCs. In short-term adhesion experiments, 10-20% of PB-EPCs and HAECs seeded onto either FN-coated glass or confluent SMCs adhered after ten minutes. Both the PB-EPCs and HAECs exhibited greater than 95% retention of the cells adhered after ten minutes of exposure to shear rates of 100 dynes/cm². Finally, the seeding density of PB-EPCs required to create a confluent layer of EPCs over SMCs was determined. Future experiments will compare the adhesion and retention rates of PB-EPCs and HAECs adhered for 24 hours over FN-coated glass and SMCs after 10 minutes or 48 hours of exposure to a physiological shear stress of 15 dynes/cm². Furthermore, the spreading of PB-EPCs and HAECs over FN-coated glass and confluent SMCs after 24 hours will be evaluated. Overall, characterization of the adhesive properties of PB-EPCs is necessary for their establishment as an alternative cell source for endothelialization of TEVGs.

Surface Projections of Titanium Substrates Increase Endothelial Cell Response to Shear Stress

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Statement of Purpose: Despite the therapeutic benefits of both mechanical circulatory assist devices and nitinol stents, problems remain with thrombosis at the blood-contacting surface. Covering these titanium (Ti) blood-contacting surfaces with a layer of endothelium would mimic the native lining of the cardiovascular system. We previously demonstrated that EPCs isolated from blood in humans and swine adhere strongly to uncoated Ti under physiological and supraphysiological shear stress, inhibit platelet adhesion and produce nitric oxide (Achneck). Surface topography and its effect on a seeded cell layer are also of particular interest in designing an endothelial implant covering, because existing Ti implants have rougher surfaces than glass slides often used in research (Jantzen). Although grooves have been studied extensively, stents and ventricular assist devices exhibit surface protrusions. In this study we tested the hypothesis that endothelial cells (ECs) have altered function on Ti surfaces with micron-size protrusions, compared to the normal roughness of Ti-covered glass surfaces, <1 nm – as evaluated in flow conditions by EC gene expression, spreading, and alignment. We further hypothesize that ECs have significantly different gene expression on the rougher surfaces, correlating to altered EC function.

Methods: Slides with evenly spaced pyramidal protrusions of 1.5 μm , 3 μm , and 5 μm height were designed for fabrication on silicon wafers. An oxide layer was first deposited, followed by masking and etching. As we have done previously (Achneck), a layer of Ti was deposited on the patterned slides and on standard glass microscope slides (Ti-glass), which served as a control. Surface roughness and peak-valley height were measured by optical profilometry (Zygo NewView 5000). ECs derived from late outgrowth umbilical cord blood-derived endothelial progenitor cells were seeded onto slides and exposed to flow at physiological (15 dyn/cm^2) shear stress for 48 hours (n=3 each surface). Cells on corresponding surfaces in static culture served as control. Medium samples were collected at the onset of flow and at 48 hours for analysis of nitric oxide (NO) production. Following flow experiments, total RNA was collected for gene expression analysis (Aurum Total RNA Mini Kit, Biorad) and portions of samples were fixed for staining and imaging. EC area, angle, and roundness were analyzed for both static and flow conditions using ImageJ software in >100 cells per sample and compared with Two-Factor ANOVA. RT-PCR was performed (iSCRIPT kit and iQ SYBR Green kit, Biorad) to evaluate expression of antithrombotic genes eNOS, COX2, and transcription factor KLF-2; GAPDH served as housekeeping gene and cells grown in culture flasks provided reference RNA.

Results: Peak-valley height of the three surfaces was found to be 4700 nm, 3000 nm, and 200 nm, respectively; manufacturing etch times led to the reduced height of the smallest features. Surface roughness of Ti-glass control was 0.73 nm. Based on this peak-valley information, local shear stress at the top of each protrusion was calculated to be increased over the average level by 26%, 17%, and 1%, for the features measured to be 5 μm , 3 μm , and 0.2 μm height, respectively (Barbee). Following 48 hours exposure to physiological flow and shear stress, cells on all surfaces significantly aligned in the direction of flow compared to static controls ($p<0.05$). Presence of the surface protrusions caused a significant decrease in the angle of alignment of endothelial cells in the flow condition compared to Ti-glass ($p<0.05$), although no significant difference in angle was observed among the feature sizes (Figure 1). Presence of the surface protrusions caused a significant decrease in the roundness of endothelial cells in the flow condition compared to Ti-glass ($p<0.05$), although no significant difference in roundness was observed due to the feature sizes. Cell area with flow was significantly reduced on the 3 μm and 5 μm features, compared to the 0.2 μm and Ti-glass surfaces ($p<0.05$). Preliminary investigation of gene expression showed upregulation of eNOS, COX2, and KLF-2 with flow for all surface conditions; however, the extent to which flow upregulated the genes varied with surface type. These preliminary studies showed expression of eNOS and KLF-2 to be greatest on the largest

features, based on the fold-change expression of flow vs. static. Conversely, the extent to which COX2 expression increased in flow vs. static samples declined with increasing feature height.

Conclusions: We show in these studies that surface roughness of Ti substrates alters typical responses to flow exhibited by ECs, including cell area, elongation, and alignment. Increased elongation, alignment, and expression of eNOS and KLF-2 correspond to higher local shear stress. These studies provide proof of concept for application of EC seeding to Ti implants of various surface roughnesses and can serve to inform implant design for endothelial coverage. Further investigation of gene expression and NO production is underway.