# Covalently Bound VEGF to Bioengineered Surfaces can Phosphorylate VEGFR-2 but it is Not Internalized

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**Statement of Purpose:** Vascular endothelial growth factor (VEGF) is known to activate proliferation, migration, and survival pathways in endothelial cells through phosphorylation of VEGF receptor-2 (VEGFR-2). VEGF has been incorporated into biomaterials through encapsulation, electrostatic sequestration, and covalent attachment, but the effect of these immobilization strategies on VEGF signaling has not been thoroughly investigated. Further, although growth factor internalization along with the receptor generally occurs in a physiological setting, whether this internalization is needed for receptor phosphorylation is not entirely clear. In this work, we aim to study covalently bound VEGF in the context of a signaling analysis. We examined whether VEGF ligand internalization was necessary for VEGFR-2 phosphorylation and downstream signaling, and evaluated the half-life of VEGF after covalent immobilization. We believe the method of growth factor incorporation into a biomaterial is an important parameter to consider when designing tissue-engineering scaffolds and is a design parameter that is typically neglected.

**Methods:** *VEGF Immobilization, exposure to cells and molecular assays*: VEGF was covalently attached to heparin functionalized gold slides through a photoactive incorporated into heparin following an oxidation reaction. This same oxidation reaction generated aldehydes that were utilized for attachment of the heparin to gold surfaces modified with PEG-alkanethiols displaying amine head groups through reductive amination. Human umbilical vein endothelial cells (HUVECs) were plated onto flexible fibronectin-modified poly-dimethoxy silane (PDMS) sheets and put in contact with the VEGF modified surface. Cell lysates were collected and then analyzed by standard Western Blot (WB) techniques. For the cell-based ELISA, cells were fixed and permeabilized, then probed with Avidin-HRP (R&D Systems), and developed with TMB substrate (Cell Signaling). *Optical tweezers*: VEGF was immobilized to heparin functionalized 3 µm polystyrene particles. Cells were plated at sub-confluence on glass bottom culture dishes. Immediately before the optical trap was used, the VEGF functionalized particles were placed in the presence of cells. The optical trap laser controlled placement of the particles next to HUVECs, and then control the oscillation and measure the rupture force.

**Results:** WB analysis showed that covalently bound VEGF (Vc) elicited an extended response of the pVEGFR-2(Y1214)/cdc42/p38 pathway, while soluble VEGF (Vs) primarily signals through pVEGFR-2(1175) residue in human umbilical vein endothelial cells (HUVECs). Our data also suggest that the covalent linkage prevents internalization of the growth factor during receptor endocytosis. Optical tweezer measurements show that the rupture force required to disrupt the heparin-VEGF-VEGFR-2 interaction increases when a covalent bond is introduced between VEGF and heparin. Importantly, by covalently binding VEGF to a heparin substrate, the stability (half-life) of VEGF is extended over three-fold. To further validate our findings we used a kinetic mathematical model to model the VEGFR-2 phosphorylation by Vc and Vs support the biological conclusions, further suggesting that VEGF is not internalized when covalently bound, and indicating that VEGF is available for repeated phosphorylation events.

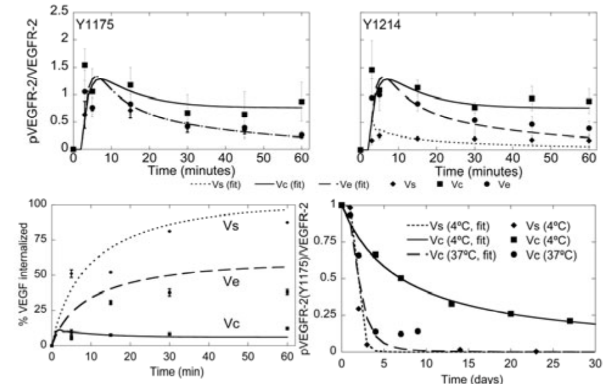


Figure 1. Quantification of WB analysis of VEGFR-2 phosphorylation at Y1175 (top left) or Y1214 (top right) by Vs, Ve, and Vc. Internalization of VEGF ligand over time when exposed as Vs, Ve and Vc (bottom left). VEGF activity half-life (ability to phosphorylate VEGFR-2) over time for Vc and Vs (bottom right).

**Conclusions:** Strategies that involve VEGF incorporation into a biomaterial range from encapsulation of the growth factor to electrostatic sequestration and covalent attachment. Some of these strategies aim to control release of the VEGF over-time. These different approaches of VEGF incorporation result in different presentations of VEGF to the cells, soluble versus matrix bound. The latter has been shown to improve vascular density and in general result in more mature blood vessels. This talk will present data that suggest that differences in signaling may result the observed differences. Perfusion is a major obstacle to implementing regenerative medicine strategy to a wide range of disease states. Biomaterial strategies that incorporate covalently bound VEGF and present the growth factor to the cell surface receptor in bound form can promote branching morphogenesis through cdc42/p38 signaling, which may lead to formation of a microvasculature that can support perfusion of the implant. Future methods should incorporate combinations of these strategies in order to form a more physiological, hierarchical blood vessel infrastructure in the engineered tissue implants.


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