POLYMER-PEPTIDE CONJUGATES AS MIMETICS OF ERYTHROPOIETIN AND VASCULAR ENDOTHELIAL GROWTH FACTOR

By

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Many cellular pathways are dependent on receptor activation by proteins which have low stability. Recombinant growth factors and cytokines used to activate these pathways are expensive to produce and rapidly denature in solution. Short peptide sequences have been developed that are able to mimic the activity of various recombinant proteins. These mimetics frequently derive from sequences present within the native protein ligand and therefore retain the ability to bind to receptors. While peptides are advantageous with regard to their stability, they typically are unable to oligomerize and effectively cluster cellular receptors resulting in a weak cellular response. To overcome this problem, we seek to develop polymer-peptide conjugates that allow for the multivalent display of peptide binding units to receptors and enhance receptor oligomerization and potentiate the cellular response. For this study, we focus on developing mimetics of Vascular Endothelial Growth Factor (VEGF) and Erythropoietin (EPO). These proteins were selected as they have well defined pathways and receptor binding as well as characterized mimetic peptides. A library of 60 polymer-peptide conjugates differing in composition and peptide valency was developed for each of the target proteins. The conjugates were screened for bioactivity, revealing several that exhibited low levels of receptor activation.
ACKNOWLEDGEMENTS

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1: Introduction

The regulation of cellular pathways is often mediated by signaling proteins such as growth factors and cytokines [1]. These proteins act via receptor-ligand binding leading to a corresponding cellular action [1]. Stimulation of these cellular pathways can be utilized in a variety of applications in the laboratory, clinical therapeutics, and in industrial cell culture. Recombinant proteins are widely used in vivo and in vitro to selectively activate cellular pathways via receptor binding and oligomerization [1]. The primary concern when working with these proteins is their characteristic low stability and high cost of production. For these reasons, there is much interest in the development of low-cost mimetics that exhibit increased stability as compared to recombinant proteins while maintaining comparable activity. The use of short peptide sequences represents reduced cost and improved stability over recombinant proteins. Recombinant proteins are produced by modified cell lines cultured in bioreactors which incur high operating costs [2]. Peptides, on the other hand, can be quickly produced with microwave synthesizers. Many peptide mimetics have short sequences and form simple secondary structures. This increases their overall stability in comparison to proteins which undergo higher degrees of fragile folding. Some mimetic peptides have similar structure to the binding domain of a protein and therefore retain the capacity to bind and activate a receptor, while others have little to no sequence homology to the native protein [3,4]. The drawback in utilizing peptide mimetics is that they are unable to effectively oligomerize receptors similar to their protein analogues, as short peptides consist of only one binding domain. This results in a reduced ability to cluster receptors and form a cellular response. The aim of this work is to overcome this issue by conjugating known mimetic peptides to polymer backbones to
allow for multivalent display of peptide binding units to cell receptors, thereby enhancing the signaling strength of these mimetics. For this study we focus on developing two sets of mimetic polymer-peptide conjugates; one set of conjugates to target the cytokine EPO, and another to target the growth factor VEGF. The goal in developing the proposed conjugate libraries is to determine if the multivalent display of peptides enhances their bioactivity as compared to monomeric peptide.

1.1: EPO and mimetic peptide EMP1

The cytokine EPO is the primary regulator of erythropoiesis [5,6,7]. EPO acts by inducing proliferation and differentiation of erythroid progenitor cells through ligand-receptor binding with the EPO receptor (EPOR) [5,6,7]. EPO also plays a role in cell survival and angiogenesis [6,8]. Upon binding to its receptor, EPO induces a conformational change that causes the extracellular domains of EPOR to dimerize and activate [7]. When EPOR is activated, it’s intracellular domain rapidly associates with Janus kinase 2 (JAK2), triggering the tyrosine phosphorylation of JAK2 [7,9].

Several cell types throughout the body naturally secrete EPO in response to hypoxic conditions so that proper blood-oxygen levels are maintained [6,7]. EPO’s ability to induce the production of red blood cells is of particular interest in therapeutics used to treat patients with anemia [6,7]. The stabilization of EPO is paramount in developing effective therapeutics. One method that has been explored is the use of small peptides capable of EPO mimicry [3,5,10]. One such peptide is EPO-mimetic peptide-1 (EMP1) [3,5,10]. The amino acid sequence of EMP1 is GGTYSCHFGPLTWVCKPQGG and contains an intramolecular disulfide bridge between the cysteine residues [5,10]. For EMP1 to effectively dimerize EPOR, two peptide monomers must associate within the binding cleft
of the receptors [3,5,10,11]. Within the binding cleft, peptide monomers form noncovalently linked homodimers, with residues of each peptide monomer participating in the binding of both receptors [10,11]. Binding of EMP1 to EPOR is primarily dependent on the amino acid residues near and within the loop formed by the disulfide bridge (Figure 1) [10,11]. The residues on the C- and N-termini do not participate in receptor binding [10,11]. The low impact of terminal amino acids on bioactivity makes them amenable to conjugation to polymers with linkers. EMP1 has exhibited enhanced mimetic activity after dimerization via C-terminal linkage of two peptide monomers [5]. For conjugate synthesis the amino acid sequence of EMP1 was modified to add a linker on the C-terminus, GGTYSCHFGPLTWVCKPQGGSS{K(N3)}. To study the effect that conjugates presenting EMP1 have in vitro, TF-1 cells were selected for use in all experiments. TF-1 cell proliferation is dependent on treatment with specific cytokines. One of the cytokines that TF-1 cells respond to is EPO.

**Figure 1** [11]: Crystal structure of EMP1 in the binding cleft formed by two EPOR. Two EMP1 monomers form symmetrical contacts with domains from both EPOR in the binding cleft. Reproduced with permission from Middleton, S. A., et al., "Shared and Unique Determinants of the Erythropoietin (EPO) Receptor Are Important for Binding EPO and EPO Mimetic Peptide."
It has been reported that dimerization of the EMP1 peptide markedly increases bioactivity. This enhancement in bioactivity with dimerization resulted in the development of a drug based on the peptide to market \([5,12,13,14]\). The drug Peginesatide (Omontys), consists of two peptides covalently linked at their C-terminus by a peg chain \([12,13,14,15]\). The amino acid sequence of Peginesatide differs from EMP1 due to the replacement of several amino acid residues, yet they share a nearly identical binding loop (Figure 2) \([13,14]\). The drug was used to treat anemia in patients with chronic kidney disease, however, it was quickly taken off the market due to adverse reactions to the drug such as immunogenic toxicity \([12,13,14,15]\). While there were serious issues with the product, the adverse effects were seemingly due to the drug formulation and not an inherent issue with the dimerized peptide \([15]\).

**Figure 2** \([14]\): Comparison of primary structures of binding loops of (a) peginesatide, (b) EMP1.

Reproduced with permission from Vogel, M., et al., "EPOR-Based Purification and Analysis of Erythropoietin Mimetic Peptides from Human Urine by Cys-Specific Cleavage and LC/MS/MS."
1.2: VEGF and mimetic peptide QK

The growth factor VEGF plays a role in several biological pathways, all of which make it a potent inductor for the formation and maintenance of vasculature [16,17]. The primary behavior VEGF regulates include angiogenesis, proliferation, migration, cell survival, and vascular permeability via expression of endothelial nitric oxide synthase (eNOS) (Figure 3) [16,17,18,19].

![Figure 3](image_url)

**Figure 3:** General schematic of the VEGF angiogenic signaling cascade.

VEGF proteins form covalently linked homodimers which allow for bivalent display of binding domains to receptors [16,21]. VEGF proteins are encoded by several genes, leading to the production of distinct signaling molecules denoted as VEGF-A, VEGF-B, VEGF-C, VEGF-D, and Placental growth factor (PlGF) [16,19,20]. Each of
these factors in the VEGF family have different specificity and binding affinities for the three VEGF receptors (VEGFR), VEGFR-1, VEGFR-2, and VEGFR-3 [16,19,20]. VEGFR-1 and VEGFR-2 are expressed in many cell types throughout the body whereas VEGFR-3 is primarily expressed and associated with the development of lymphatic vasculature [16,17,19,20,22,23]. As VEGFR-1 and VEGFR-2 are expressed throughout most of the vasculature of the body, targeting them is more therapeutically significant as compared to VEGFR-3. VEGF-B and PI GF specifically bind to VEGFR-1 [19]. VEGF-C and -D are able to bind VEGFR-2 and -3 [19,20,22,23]. Of the VEGF family of proteins, only VEGF-A is capable of binding both VEGFR-1 and VEGFR-2 as well as heterodimers of these two receptors [19,20]. Our development of a protein analog seeks to mimic VEGF-A, due to the unique binding activity it exhibits.

As a result of alternative splicing of VEGF mRNA, there are several isoforms of VEGF-A [19,20,24]. Of the splice variants of VEGF-A, three isoforms are expressed more frequently than the rest; VEGF_{121}, VEGF_{165}, and VEGF_{189} [20]. The ability for certain VEGF isoforms to bind heparin yields an increase in association with cell surface receptors and extracellular matrix (ECM), enhancing the potency of these growth factors [20]. Heparin binding also serves to prolong the activity of VEGFs by protecting them from degradation [20]. VEGF_{121} lacks the amino acid sequence that allows for heparin binding, resulting in it being a weak mitogen [20,24]. VEGF_{165} contains 44 more amino acid residues than VEGF_{121} which allows it to bind heparin and enhance its binding to receptors [20,24]. On the other hand, VEGF_{189} has the longest amino acid sequence, containing 24 more amino acids than VEGF_{165} [20]. The additional 24 amino acids produce further enhancement to the proteins association with surface receptors and ECM, however, those
residues also obstruct the binding domains of the protein resulting in reduced mitogenicity as compared to VEGF\textsubscript{165} [20]. VEGF\textsubscript{165} is the isoform often used in cell culture and is typically the variant referred to in research related to angiogenesis.

The complexity of the relationship between VEGF and its receptors/co-receptors differs from EPO, which binds to only one receptor (1:2 ligand:receptor stoichiometric ratio). In developing a suitable mimetic for VEGF, there exists the added obstacle of finding a peptide capable of binding multiple VEGFR targets.

The binding of VEGF to VEGFR-1 has been thoroughly studied and has been shown to depend primarily on the interaction between VEGF and extracellular domains 2 and 3 of VEGFR-1 (VEGFR-1\textsubscript{D2} and VEGFR-1\textsubscript{D3}) [24,25]. This dependence has led to the development of several VEGF mimetic peptides whose amino acid sequence have been derived from regions of the VEGF protein involved in binding these domains [4,25,26,27]. Of these mimetic peptides, peptide QK is of particular interest for use in a polymer-peptide conjugate as it is stable, well characterized, capable of binding and activating VEGFR-1 and -2 and has been shown to retain its mimetic activity when immobilized [4,28,29,30]. Peptide QK was derived from the helical structure found in residues 17-25 of VEGF [4,28]. The peptide forms an α-helix with the amino acid sequence KLTWQELYQLKYKGI, capped with an amide on the C-terminus and acetylated on the N-terminus [4]. QK has been functionalized onto many different materials using either the C- or N- termini as linkage sites, while still retaining its mimetic activity [29,30,31]. As QK does not display any binding dependence on its termini, the N-terminus was used as the linkage site for polymer conjugation. The modified amino acid sequence used for conjugate synthesis is, \{K(N3)\}GGKLTWQELYQLKYKGI. To study the effect that conjugates presenting QK
have \textit{in vitro}, the primary cell line, Human Umbilical Vein Endothelial Cells (HUVEC) were selected for use in all experiments.

1.3: PET-RAFT Polymerization

Utilization of photoinduced electron/energy transfer–reversible addition-fragmentation chain-transfer (PET-RAFT) polymerization allows for the ability to easily generate large libraries of polymers for conjugation with our selected peptides [32]. PET-RAFT is a type of oxygen tolerant polymerization, that requires a catalyst such as zinc tetraphenylporphyrin (ZnTPP) and is activated by light (Figure 4) [32]. This type of polymerization allows for high control over the reaction, yielding precise molecular weights with low polydispersity [32]. Synthesis of polymers in this way allows for low volume reactions in 96-well plates while maintaining complete conversion to the desired product [32]. Prior to the development of robust oxygen tolerant polymerization techniques, synthesis of large polymer libraries would have taken a lot of time as well as required special equipment so that each polymerization reaction could occur in a deoxygenated environment [33]. The degree of polymerization (DP) is the number of monomer units within a polymer. In a polymerization reaction DP is dependent on the ratio of the concentration of chain transfer agent (CTA) to the concentration of monomers present in solution [32].
Figure 4 [32]: Mechanism of PET-RAFT polymerization involving ZnTPP, yielding polymers of varying architecture. Reproduced with permission from Gormley et al., “An Oxygen-Tolerant PET-RAFT Polymerization for Screening Structure–Activity Relationships.”

2: Methods

2.1: Peptide Synthesis

The QK and EMP1 peptides used in this study were synthesized by High Efficiency Solid Phase Peptide synthesis (HE-SPPS) using a Liberty Blue automated peptide synthesizer (CEM Corporation). Custom peptides were designed, modified with an added lysine-azide linker on the N-terminus of peptide QK and the C-terminus of peptide EMP1. The synthesizer builds the peptide chain from the C-terminus to the N-terminus, starting with a Rink amide resin. Amino acids in the peptide sequence are then added one at a time until the full chain is produced. For the QK peptide, the N-terminus was acetylated by addition of a mixture of dimethylformamide (DMF)/acetic anhydride/N,N-
diisopropylethylamine (DIEA) (92.5:5:2.5, v/v/v), for 10 minutes. Successful capping of the peptide was checked by performing a Ninhydrin test, which determines the presence of a free amine group. After capping the QK peptide, both peptides were cleaved from the resin. The peptides are allowed to react in the cleavage solution for 2-5 hours with constant air bubbled through the solution. The cleavage solution used consists of a mixture of trifluoroacetic acid (TFA)/ triisopropylsilane (TIS)/water (95:2.5:2.5, v/v/v), with dithiothreitol (DTT, 2.5% w/v to maintain a reduced state and prevent the oxidation of tryptophan present in the peptides. After the peptide is separated from the resin, the cleavage solution is collected and the resin is rinsed with dichloromethane (DCM). A rotary evaporator is used to reduce the volume of the solution containing the peptide. The remaining solution is then added dropwise into cold diethyl ether to precipitate the peptide. The peptide is then centrifuged so that the diethyl ether can be aspirated, leaving the peptide behind. The peptides then undergo vacuum desiccation to remove any remaining ether.

The peptide was then purified by flash chromatography using a CombiFlash system (Teledyne ISCO). The peptides are dissolved in a water/TFA mixture (99.9:0.1 v/v) and loaded onto the column followed by a gradient of water/acetonitrile. Fractions containing peptide were rotary evaporated to remove the acetonitrile and lyophilized. Once lyophilized the QK peptide is ready for use, however, the EMP1 peptide needs to be cyclized. EMP1 is cyclized between residues 6 and 15 by reconstitution in a 10 mM borate buffer (pH 8.5) and stirred for 24 hours with bubbling. Peptide was then purified and lyophilized again.
2.2: Polymer-Peptide Conjugate Synthesis

PET-RAFT was utilized to polymerize monomers with acrylic acid N-hydroxysuccinimide (NHS) to form our polymer backbones [32]. Following polymerization, dibenzocyclooctyne-amine (DBCO-NH$_2$) was then incorporated into the polymer backbones. To determine the success of DBCO incorporation, the polymers are measured by absorbance reading at 295nm, which is the UV-vis peak for DBCO. This measurement is compared to a standard curve of known DBCO concentrations. Once the DBCO content on the polymer backbones is determined, an equal amount of peptide is added to the polymers in solution. Peptides are synthesized with an azide modified lysine linker on one of their termini and the DBCO attached to the backbones contain an alkyne, which allows for the use of strain promoted alkyne-azide cycloaddition (SPAAC) to click the peptide on to the polymer backbone.

The polymer backbones used in this study consisted of different combinations of 2-Hydroxyethyl Acrylate (HEA), Methoxy PEG acrylate with a molecular weight distribution of approximately 350 Daltons (MPEG300), Polyethylene glycol Monomethylether Monomethacrylate (PEGMA), Methyl Acrylate (MA), Methyl Methacrylate (MMA), Butyl Acrylate (BA), and NHS monomers with a degree of polymerization of 200 monomer units (Table 1). NHS incorporation into polymer backbones was varied between 5%, 7.5%, and 10% of total monomer units, yielding conjugates which each displayed between 10 and 20 peptides.
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Polymers were purified by centrifugation in desalting columns, which act like size exclusion chromatography columns, and diluted into Dimethyl sulfoxide (DMSO). The conjugates are highly soluble in the organic reagent DMSO, though care must be taken in conjugate testing as the reagent is cytotoxic. Conjugates were prepared in polymerase chain reaction (PCR) plates as they have low volume wells and retain the same well spacing as 96-well plates allowing for easy transfer of conjugate samples to assay well plates using standard multichannel pipettes.

Characterization of synthesized polymers was conducted by Gel permeation chromatography (GPC) and multi angle light scattering (MALS). GPC measurements are dependent on the results of elution time of PEG standards with known molecular weights. Therefore, accuracy of GPC data is reliant on the similarity of the sample polymer to PEG chains. For this reason, MALS measurements are also taken for polymer samples. The GPC and MALS detectors are placed in series and preformed using DMF and LiBr as the eluent. The polymers are not directly tested through this system as NHS has issues due to interaction with the GPC column and can skew GPC data, which is time dependent on the release of molecules from the column, as well as damage the column. Instead representative polymers with no NHS inclusion are characterized to determine the performance of the polymerization reactions. An example of molecular weight data for representative polymers with DP 200 including, a homopolymer of HEA (representative of polymer backbones #25-27 in Table 1) and a heteropolymer containing an equal number of monomer units of HEA and mPEG300 (representative of polymer backbones #1-3 in Table 1).
1), can be seen below in Figure 5. The synthesized polymers had low polydispersity indexes (PDI) at approximately 1.1 as determined by GPC and MALS data (Table 2).

Figure 5: Molecular weight data of homopolymer HEA (representing polymer backbones #25-27) and heteropolymer HEA-mPEG300 50%-50% (representing polymer backbones #1-3), determined by GPC.

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<th>MW&lt;sub&gt;MALS&lt;/sub&gt; (Da)</th>
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Table 2: Molecular weight and PDI of homopolymer HEA and heteropolymer HEA-mPEG300 50%-50%, determined by GPC and MALS.

2.3: Cell Culture Method

TF-1 cells were purchased from ATCC and grown in RPMI 1640 growth medium (10% Fetal Bovine Serum (FBS), 1% Penicillin/Streptomycin, 2mM L-Glutamine) (VWR). TF-1 cultures were maintained between $3\times 10^4$ and $5\times 10^5$ viable cells/mL in suspension with media being replaced every 3 days as recommended by the supplier. Donor
pooled HUVEC were purchased from Lonza Bioscience and grown in Endothelial Growth medium-2 (EGM-2) (EBM-2, 10% FBS, hEGF, hFGF, VEGF, R3-IGF-1, ascorbic acid, GA-1000, heparin, hydrocortisone). HUVECs were seeded in flasks at a density of 5,000 cells/cm² and harvested at 75% - 85% confluency for experimentation. Experiments involving HUVEC were conducted using cultures between passages 3-7, as cells at high passage numbers (i.e. passage number 8+) can become senescent and lose their cell line specific properties. Both cell lines, TF-1 and HUVEC, were cultured in humidified incubators, at 37°C and 5% CO₂.

2.4: Proliferation-based Assays

Cellular proliferation assays were used to screen QK and EMP1 conjugates for potential bioactivity. Conjugates that were able to induce proliferation underwent further testing with a wider range of concentrations and with DMSO concentrations under 0.01% v/v. Conjugates were added to media near their solubility limits (approximately 150 nM for EMP1 conjugates and 1 μM for QK conjugates) with final DMSO concentrations of 0.5% v/v.

2.4.1: VEGF Proliferation Assay

HUVEC were plated (10,000 cells/well) in 96-well plates, coated with 0.1% gelatin solution, and allowed to adhere and recover for six hours. Wells were then treated with starvation media (EBM-2 supplemented with 0.25% FBS and 0.75 Units/mL heparin sulphate) containing serial dilutions of either VEGF, QK, or conjugates and allowed to incubate for 72 hours. Complete media containing resazurin sodium salt was added to wells and allowed to incubate for 3 hours. Resazurin is reduced to resorufin, which is highly
fluorescent. The indicator is reduced in proportion to the number of viable cells in a well. Reduction of resazurin was quantified via fluorescence measurement (excitation/emission: 555/585nm).

2.4.2: EPO Proliferation Assay

TF-1 cells were plated (25,000 cells/well) in 96-well plates in 50 µL. An equal volume of media containing serial dilutions of either EPO, EMP1, or conjugates and allowed to incubate for 72 hours. Resazurin sodium salt suspended in phosphate buffered saline (PBS) was then added to all wells and allowed to incubate for 6 hours after which resazurin reduction was quantified by fluorescence reading at 555/585 nm excitation/emission wavelengths and using a cutoff filter of 570 nm.

2.5: Angiogenesis Assay

HUVEC, suspended in starvation media, were plated (25,000 cells/well) in 96-well plates coated with 10 mg/mL growth factor reduced (GFR) Matrigel. Matrigel aliquots were thawed overnight on ice in a 2-8°C refrigerator. 50 µL of Matrigel was added to each well, after which well plates were spun at 100xg for 3 minutes in a centrifuge precooled to 4°C and then placed in 37°C humidified incubators for 30 minutes prior to use. Wells were treated with test substances at the time of plating. Images were taken after 16 hours, to quantify total tubule length.

2.6: Migration Assay

HUVEC were grown to confluency in 24-well plates, coated with 0.1% gelatin solution. A scratch was made in the confluent monolayers with a P-200 pipette tip, to simulate a wound, after which the wells were washed with PBS to remove cells lifted from
the culture surface. The wells were then treated with test substances. Images were taken at 0 hours and 18 hours and processed using ImageJ to quantify the wound closure.

2.7: Statistical Analysis

Conjugate data was subject to ANOVA analysis where appropriate to determine if results exhibited statistical significance as compared to the corresponding negative control used. ANOVA analyses were performed using OriginPro software. All statistical significance was determined at the 0.05 level. Dose response curves were generated using a sigmoidal dose response model.

3: Results and Discussion

3.1: Cell Line Selection

To determine the efficacy of the produced polymer-peptide conjugates for use as protein mimetics, robust assays needed to be developed. Cellular responses to the conjugates need be quantified and compared against the bioactivity of recombinant proteins as well as to monomeric peptides. Both EPO and VEGF are capable of inducing proliferation, which is simple to detect and amenable to a high throughput assay format. As a relatively high number of conjugates will be tested, proliferation will be the primary assay metric studied. The first step in developing these assays is to select the cell lines. For the EPO mimicking conjugates, the TF-1 cell line was selected. TF-1 cells are erythroleukemia cells that grow in suspension and are derived from a human bone marrow sample. TF-1 cell proliferation is dependent on the addition of cytokines, such as EPO, to their normal culture media. If any of the conjugates are bioactive and mimic EPO, the TF-1 cells should exhibit some level of increased proliferation.
Selecting a cell line to test the VEGF mimicking conjugates was not as straightforward. Endothelial and epithelial cells express VEGFRs, however, their proliferation is not dependent on VEGF and can occur due to other factors including proteins found in FBS. These cells are not typically very robust and can be highly sensitive to changes in their culture environment. Primary endothelial cell lines are only capable of undergoing a limited number of population doublings before losing their endothelial phenotype, reducing their angiogenic activity or becoming quiescent [34]. Immortalized endothelial cell lines and other cells modified to express VEGFR overcome the limitation in population doublings, however, these cell lines have low angiogenic potential. In order to fully understand the capacity for the QK conjugates to mimic VEGF, assays investigating cellular migration and tubule formation must be utilized in addition to proliferation assays. Ideally, the same cell line should be utilized for all assay types. Modified and immortalized cell lines do not maintain the same capacity to form tubules and migrate as compared to normal endothelial cells and therefore do not meet the requirements for this study [34].

When it comes to primary endothelial cells there are many options including large and small vein macrovascular cell lines cell lines as well as cells derived from specific organs [34]. For this study, HUVEC, a large vein cell line was selected as it is the classic cell line used to study angiogenesis and has been used to study the effects of the QK peptide previously [28,34]. HUVEC present both VEGFR-1 and -2 surface receptors and are a widely used cell type for the study of angiogenesis. Being that HUVEC are primary cells, there exists differences in the angiogenic capacity of cells from different sources. To overcome the issue of donor cell source, all experiments can either be characterized using
cells from the same source, or donor pools. Donor pooled cells are cells from multiple sources that have been mixed together. Doing so mitigates the difference between cell sources as cells that have higher mitogenicity will proliferate and overtake more quiescent cells in culture. For our study, donor pooled cells were used in all experiments.

3.2: Proliferation Assay Development

There are several ways to quantify cellular proliferation, all of which range in throughput, sensitivity, and technical rigor. Cell proliferation can be measured by simply counting the number of cells present at the end of an experiment, either visually or via DNA content. DNA production and expression is tightly regulated and expression is directly proportional to cell number. Therefore, methods to quantify DNA can be used to quantify cell number. Measuring cell number in this way has the advantage of not depending on the metabolic state of the cells which is a common problem for other assays. Assay kits for quantifying DNA content can be expensive with only marginal gains in sensitivity over other methods.

Proliferation can also be measured by live cell staining with dyes, such as crystal violet, whose absorbance decreases with each subsequent cellular division, thereby relating cellular absorbance to the number of population doublings that have occurred. Stains used in this way remain in the cell, however, cellular concentration decreases due to the dye being split between the cytoplasm of daughter cells after cellular division occurs. Flow cytometry is typically used to quantify cellular absorbance and is a low throughput method, especially for adherent cells which must first be lifted off of their culture surface before flow cytometry can be conducted.
The last method for measuring proliferation is via cellular viability assays, which include reagents such as tetrazolium salt (MTT) and resazurin [35]. Cell viability assays depend on the metabolic state of the cells, where a reagent is metabolized proportionally to the number of cells. MTT is cytotoxic and is reduced by cells resulting in the production of formazan crystals, which must then be solubilized before an absorbance reading can be taken [35]. The solubilization of the formazan crystals can be achieved through the addition of various lysing solutions including Sodium Dodecyl Sulfate (SDS). In comparison, resazurin based compounds such as alamarBlue and PrestoBlue are non-cytotoxic and can be used for determining the viability of live cells. Resazurin is a blue dye that is reduced by cells into the fluorescent product, resorufin, which is pink in color and can be measured by absorbance or fluorescence (Figure 6) [36]. The addition of resazurin sodium salt alone to culture media will result in the metabolization of the compound by cells. Resazurin containing solutions such as alamarBlue and PrestoBlue help stabilize and quicken the reduction of the compound by cells.

![Figure 6](image)](image)

In this study MTT was initially used to quantify cellular proliferation, however, resazurin provides several benefits and was later used to develop the assay and test the polymer-peptide conjugates. Both of these dyes allow for high throughput testing of samples, with resazurin having a slight advantage not requiring the added step of solubilization. The decision to switch to resazurin dye was a composite of several factors, the greatest of which was the performance of HUVEC metabolism of MTT dye. Resazurin also allows for the ability to check cell viability at multiple time points without the need for additional assay replicates. The use of the compound also leaves the door open for future tests to be run in parallel within the same well plate, such as the use of caspase indicators, which can be used to quantify cellular apoptosis, given that the fluorescence or absorbance of the indicators does not overlap with the wavelengths used to measure resorufin.

3.2.1: EPO Proliferation Assay

A proliferation assay suitable to test the EMP1 conjugates must exhibit reproducible positive and negative control values, with a sufficient range between them to allow for the visualization of EMP1 activity. Experiments using MTT dye and a cell seeding density of 10,00 cells/well yielded ED$_{50}$ curves for EPO (Figure 7) but were unable to visualize any significant activity from the EMP1 peptide. Using resazurin as an indicator did not yield a significant difference in the ED$_{50}$ of EPO seen in TF-1 cells, fluorescence measurements utilizing resazurin also did not reveal any difference between the negative control and the EMP1 peptide.
Figure 7: Comparison of cell viability quantification via MTT vs. resazurin. TF-1 cells were plated at 10,000 cells/well in 96-well plates and were exposed to serial dilutions of EPO. (A) Dose response curve using MTT dye as an indicator of cellular viability ($R^2=0.97$). (B) Dose response curve using resazurin dye as an indicator of cellular viability ($R^2=0.99$). The curve fit for both (A) and (B) is significantly better than the fit of the function $y=\text{constant}$. ($p<0.05$). $n=3$.

In the literature the EMP1 peptide has been shown to induce proliferation in TF-1 cells, albeit with significantly lower activity as compared to EPO [3,5,10]. As no significant difference was found, in this trial of the assay, between the negative control and the EMP1 peptide, the assay must be modified to increase the signal generated by the cell viability indicators. If any difference in cell number exists between the negative control and EMP1,
it is possible that the indicator dye is not sensitive enough to allow us to visualize that difference. Increasing the cell seeding density would serve to enhance any difference in bioactivity between samples as increasing the initial cell count should lead to an increase in the final cell count by the same factor, while the sensitivity of the indicator dye stays the same. At the same time, the cell seeding density cannot be too high as TF-1 cells must be cultured within a certain range of cell density in order to behave normally. The optimal cell concentration range for TF-1 cells as recommended by the supplier, ATCC, is between $2 \times 10^4$ - $5 \times 10^5$ cells/mL. If the seeding density is too high, the cells may also consume all of the nutrients in the media and undergo apoptosis before the assay is concluded. Keeping this in mind, the assay method was tested again using a cell seeding density of 25,000 cells/well in 96-well plates, which resulted in the ability to visualize a response to EMP1 (Figure 8). It was determined by ANOVA analysis that all concentrations of EMP1 depicted in Figure 8 were significantly different from the negative control at the 0.05 level. Increasing the cell density to 25,000 cells/well resulted in a shift of the ED$_{50}$ of EPO to a slightly lower concentration (Figure 9) which may be a result of metabolic changes in the cells now that they are being seeded within their optimal concentration range.

EPO has an ED$_{50}$ at approximately 20 pM and a bioactivity plateau at approximately 1 nM [3]. EMP1 achieves comparable maximum bioactivity to EPO, but with a plateau at approximately 100μM and an ED$_{50}$ at 400 nM [3]. The range of concentrations EMP1 was tested at for this assay exhibited a low range of response values between 35-45% of the maximal response to EPO making a determination for the ED$_{50}$ difficult. At the dose used in this assay, the response to EMP1 was expected to be closer to 100% of the maximal response to EPO as is reported in the literature [3]. The disparity in
bioactivity exhibited by EMP1 may be due to differences in the transfected FDCP-1 cell line used in the literature and the TF-1 cells used in this study [3].

Figure 8: Normalized response to EMP1 peptide. TF-1 cells were plated at 25,000 cells/well in 96 well plates. Cells were treated with serial half dilutions of peptide with the maximum concentration being 12.5 µM. 100% represents the positive control, TF-1 cells treated with 10 Units/mL EPO, and 0% represents the negative control, untreated TF-1 cells. All concentrations depicted are significantly different from the negative control (p<0.05). n=3.
Figure 9: Dose response of EPO using resazurin as an indicator. TF-1 cells were plated at 25,000 cells/well in 96-well plates and were exposed to serial dilutions of EPO. The curve fit depicted is significantly better than the fit of the function $y=\text{constant}$ ($R^2=0.98$). ($p<0.05$). $n=3$.

3.2.2: VEGF Proliferation Assay

There are several challenges in developing a proliferation assay for VEGF that did not arise in the development of the EPO assay, as HUVEC cells are more sensitive to their environment and media conditions. There are many parameters that need to be optimized to ensure that the HUVEC cells are healthy enough to respond to VEGF while not being exposed to any factors that would result in significant non-VEGF dependent proliferation. Setting this ideal condition for the cells is very much a balancing act.

Unlike TF-1 cells dependence on EPO for proliferation, HUVEC undergo proliferation when exposed to a variety of proteins and growth factors. The normal media conditions for culturing HUVEC include EGF and FGF, among other growth factors, which are potent inducers of proliferation. Within a proliferation assay, many of the normal protein supplements must be excluded from the culture media so that VEGF dependent proliferation is not masked by other sources and so any proliferation that occurs can be attributed to the changes in VEGF concentration. Starving the cells of their normal media supplements puts the cells under stress for the duration of the assay. If the stress is too high, the cells will lift from the culture surface and undergo apoptosis.

Normal HUVEC culture media also contains several nonprotein supplements including heparin sulphate, hydrocortisone, and ascorbic acid. Heparin is included in the media used for the assay as it serves to maintain the VEGF concentration over the course of the assay. Hydrocortisone increases cell sensitivity to VEGF, however, the cellular
response to VEGF is already expected to be significantly greater than that of the QK peptide. Therefore, it is not needed for this assay. Ascorbic acid is an antioxidant which reduces the stress on cells, however, antioxidants are also capable of reducing resazurin. Therefore, ascorbic acid must be excluded from the assay to use resazurin as an indicator.

HUVEC are cultured using a relatively low concentration of FBS (2%), however, even at that concentration FBS can contain high enough levels of VEGF or other growth factors to induce proliferation. To mitigate the proliferative effect of FBS on HUVEC, the serum concentration must be reduced to the minimum possible level while still maintaining cell viability over the course of the assay. In addition to other factors, the optimization of cell seeding density is important as HUVEC experience cell-cell contact inhibition of proliferation when they reach a confluency of 70% or greater [34]. To summarize, the parameters of this assay that require optimization are cell seeding density, method of serum starvation/serum concentration, and determination of required starvation media supplements.

Initial experiments were conducted using a procedure from Finetti F., et al., as a reference [28]. The procedure calls for HUVEC to be plated at 1,200 cells/well in 96-well plates coated with poly-D-lysine, followed by 24-hour incubation with starvation media consisting of EBM-2 supplemented with 0.1% heparin and 0.1% bovine serum albumin (BSA) [28]. The procedure used for the first trial of the assay differed from the reference procedure in that the 96-well plates used were uncoated and the detection method employed was MTT instead of CyQUANT NF which quantifies DNA content. It was found that under these conditions the HUVEC did not survive and were no longer metabolically active after 24, 48, or 72-hour treatment with VEGF. Low cell adherence was also observed after 24-
hour incubation in starvation media. HUVEC under normal conditions adhere and spread out over a culture surface, making it easy to distinguish between healthy cells and dying cells as seen below (Figure 10).

![Figure 10: Comparison of A) healthy HUVEC at confluency and B) HUVEC that have lifted from the culture surface and are no longer viable.](image)

The result of the first trial assay showed no significant difference between the negative control and the positive control (treatment with VEGF). The average absorbance at 570 nm for the controls was very close, with the values being low, likely due to the low cell viability. This result indicates that plate coating may be vital to the success of the assay. HUVEC growth and survival is dependent on surface adherence and coating the well plates may increase adherence and reduce the stress the cells experience. Alternatively, the cell seeding density may be too low, resulting in an inability to visualize any difference between the controls due to the detection method. The next assay trials included multiple cell seeding densities, $1.2 \times 10^3$, $5 \times 10^3$, and $10 \times 10^3$ as well as being plated in complete EGM-2 media and allowing the cells to recover from passaging for 24-hours in the well plates before incubation with starvation media. $10 \times 10^3$ cells/well is approximately the maximum cell seeding density where the cells are still below 70% confluency after adherence, $5 \times 10^3$ cells was tested as an intermediate cell density. These changes resulted in no change in the
ability to visualize a VEGF response. The assay was run again with serial dilutions of complete media in starvation media instead of VEGF to determine which part of the assay is resulting in low cell viability. Using complete EGM-2 media as a positive control to test if the cells are surviving the 24-hour serum starvation period. The result shows a low range of absorbance values, however there is a significant difference between the untreated cells and cell treated with complete media (Figure 11).

Figure 11: Proliferation assay using complete EGM-2 media as a positive control with several different cell seeding densities, A) 1,200 cells/well, B) 5,000 cells/well, C) 10,000 cells/well. Y-axis starts at negative control value in each graph. The curve fit for (A) 1,200 cells/well is not significantly better than the fit of the function $y=\text{constant}$ ($R^2=0.90$), ($p<0.05$). Curve fits for both B) ($R^2=0.95$) and C) ($R^2=0.97$) is significantly better than the fit of the function $y=\text{constant}$, ($p<0.05$). n=3.
The relatively low absorbance values even for the cells treated with 100% complete media indicate that there is significant cell death from the 24-hour serum starvation. The range of absorbance values seen is low with all cell densities treated with complete media, so without further adjustment to the procedure visualization of a VEGF response would be unlikely. The confluence of the cells after 72-hour treatment with complete media did not surpass 70%, as this seeding density yielded the highest range of values, it will be used in future assay trials.

To reduce the stress on the cells during serum starvation and over the course of the VEGF treatment period, gelatin was used to coat the well plates. 50 µL of 0.1% gelatin solution was added to each well of 96-well plates and incubated at 37°C for one hour before cells were seeded. The detection dye was also changed to resazurin as measuring the fluorescence values should lead to a higher range of values as compared to MTT absorbance. As resazurin is not cytotoxic, it may also reduce the stress on the HUVEC which are already sensitive due to lack of serum and nutrients. BSA concentration was tested in parallel to determine if potential nutrient exhaustion could be mitigated. Making these modifications allowed for visualization of a VEGF response but an accurate ED₅₀ curve due to high standard deviations (Figure 12).
Figure 12: HUVEC proliferation assay, resazurin fluorescence normalized to VEGF positive control. Comparison of starvation media BSA concentrations between 0.1% and 1%. 100% represents the positive control, HUVEC treated with 25 ng/mL VEGF, and 0% represents the negative control, untreated HUVEC. 

n=3

This method yielded the first response seen from VEGF, although further adjustments must be made to the procedure to lower the standard deviations seen and produce an ED$_{50}$ curve. It was also discovered that increasing the concentration of BSA in the media resulted in decreased adhesion of HUVEC cells. As increasing BSA concentration did not have the desired effect of increasing cell survival by means of providing proteins for the cells to metabolize, a switch to a low concentration of FBS (0.25%) will be made.
Figure 13: HUVEC proliferation assay, starvation media supplemented with 0.25% FBS. 100% represents the positive control, HUVEC treated with 25 ng/mL VEGF, and 0% represents the negative control, untreated HUVEC. The curve fit is significantly better than the fit of the function \( y = \text{constant} \) (\( R^2 = 0.98 \)).

\( (p<0.05), n=3 \)

The ED\(_{50}\) for VEGF tested on HUVEC is between 1-6 ng/mL, using this method we can reproducibly generate an ED\(_{50}\) in this range as seen above (Figure 13). The next step is to determine the dose response of the monomeric QK peptide. Having a baseline of activity will aid in determining if the conjugation of the peptide results in retention or enhancement of bioactivity. Using the same assay protocol, QK was tested against VEGF, the peptide itself showed low activity with no clear dose dependence (Figure 14). Preforming an ANOVA on the various QK dilutions yielded no significant difference between the QK dilutions and the negative control, except for at 0.78 ng/mL, however the significance at this dose was not reproducible.
Figure 14: HUVEC proliferation assay, starvation media supplemented with 0.25% FBS. 100% represents the positive control, HUVEC treated with 25 ng/mL VEGF, and 0% represents the negative control, untreated HUVEC. Dose response fit curve ($R^2=0.95$). Maximum concentration of QK, 100 ng/mL.

(p<0.05). n=3

The peptide QK has been seen in the literature to have significant bioactivity at 100 ng/mL on par with VEGF [28]. However, that activity is not seen in the assay results. Increasing the FBS concentration yielded no change in assay results. The discrepancy in activity may be due to the modification made to the peptide to include the lysine azide linker on the N-terminus. While the QK peptide does not exhibit any binding dependence on the N-terminus, it has been reported that binding of the peptide is dependent on the helical structure it forms [29,30,31,37]. The folding process of the QK peptide begins at the N-terminus, and reduction of the stability at that end of the peptide can disrupt the folding into its helical structure [37]. In the literature, it has been reported that QK retains its bioactivity with N-terminal modifications [30,31]. While the issue likely is the result of cellular stress due to the assay procedure, an unmodified QK peptide was synthesized and
tested under the same conditions, which also did not reveal any significant bioactivity (Figure 15).

**Figure 15:** Comparison of unmodified QK to QK synthesized with lysine azide linker on the N-terminus (QK-azide). HUVEC proliferation assay, starvation media supplemented with 0.25% FBS. 100% represents the positive control, HUVEC treated with 25 ng/mL VEGF, and 0% represents the negative control, untreated HUVEC. n=3.

Further attempts to improve the QK response were made by testing combinations of the following parameter changes; changing the length of cell treatment with VEGF/ QK, shortening the serum starvation period, shortening the cell recovery period, FBS concentration, and gelatin coating versus no coating. These changes were meant to test the cells under either harsher or more ideal conditions to see if peptide response improved while maintaining an accurate ED$_{50}$ for VEGF. Unfortunately, none of these modifications to the procedure achieved this goal.

Instead of focusing on visualizing a QK response, effort was made to adjust the assay procedure to simplify it for high throughput testing of conjugate samples. Under the
current assay procedure, cells are plated in complete media and 24 hours after seeding, complete media is aspirated, cells are washed with PBS and starvation media is added. After 24 hours of serum starvation, the starvation media is aspirated and sample treated media is added. This procedure takes five days to test complete the assay, from cell seeding to addition and incubation with resazurin, and requires several aspiration steps. Aside from the fact that instances of media aspiration should be minimized as the HUVEC are already under significant environmental stress, daily media changes can be cumbersome on a large scale due to a high number of samples. The EPO assay is simple, the cells are plated in 50 µL of media and 50 µL of sample treated media is added immediately (at 2x sample concentration). To simplify the VEGF assay, the serum starvation period was removed and cells would no longer be seeded in complete media and allowed to recover for 24-hours. Instead, cells would be plated in 50 µL of starvation media supplemented with 1–2% FBS and given three hours to recover. At this time, 50 µL of sample treated media was added (at 2x sample concentration) containing no FBS, resulting in a final FBS concentration of 0.5–1% over the course of the treatment period. This procedure resulted in visualization of a significant response to QK at several concentrations (Figure 16). The maximum concentration of QK tested was 100ng/mL, and while an ANOVA analysis revealed that some of the top concentrations did not show a significant response, several concentrations in the dilution series did. It is unknown as to why QK appears to have lower activity at the top concentrations tested. The assay was repeated to determine if the same trend would occur if the dilution series started at a higher concentration of peptide, the result displayed the same trend. Plating the cells in 1% FBS exhibited decreased standard deviations in the data as compared to plating with an FBS concentration of 2%, as well as yield higher
concentrations of QK that exhibit statistical significance. Having demonstrated reproducibility of the assay controls, the next step is to test the conjugates.

**Figure 16:** HUVEC proliferation assay, starvation media supplemented with 0.25% FBS. 100% represents the positive control, HUVEC treated with 25 ng/mL VEGF, and 0% represents the negative control,
untreated HUVEC. All of the concentrations depicted are significantly different from the negative control, 
\( p < 0.05 \). \( n=3 \).

It has been reported in the literature that monomeric QK peptide at a concentration of approximately 50 ng/mL is able to induce cellular proliferation at a comparable level to VEGF at a concentration of 25 ng/mL [28]. While no specific ED\textsubscript{50} is reported for QK, results in the literature suggest that QK exhibits approximately 40% of the maximal response to VEGF at 10 ng/mL and 50% somewhere between 10-25 ng/mL [28]. The top end of concentrations tested, 25-100 ng/mL, are significantly lower than is reported in the literature, however the result at 10 ng/mL matches what is reported for QK. While there is some disparity in measured bioactivity to that of the literature, this procedure also produced the highest response to QK out of all assay modifications and therefore was used to screen conjugate samples.

### 3.3 Effect of EMP1 Conjugates on Cellular Proliferation

EMP1 conjugates were synthesized at a range of maximum concentrations, approximately between 0.75-2 mM, as can be seen in Table 1. For simplicity, the same volume for each conjugate sample was transferred from PCR plates to assay well plates leading to slight variations in the concentration different samples were tested at. Conjugate samples were added to media at 1% v/v, after mixing at a 1:1 ratio with the media. Final DMSO concentration of the assay was 0.5% v/v. The EMP1 conjugates exhibited low media solubility, meaning that the conjugates had to first be diluted in DMSO. The smallest dilution factor at which all conjugates appeared soluble in media was a 128x dilution, making the highest starting concentration among samples approximately 150 nM. A conjugate dilution series consisting of five dilutions was prepared and tested.
While many of the EMP1 conjugates resulted in cell viability either at or below the level of the negative control, a subset of conjugates did appear to have a positively trending response with increasing concentration including conjugates #25, 26, 38, 39, 41, 42, 51, 54, and 57 (Figure 17). As these conjugates exhibited some level of bioactivity and appeared to be dose dependent, they were tested again with a reduced DMSO concentration of 0.05%. The model used to generate dose response curves tries to fit the collected data to a sigmoidal curve. In the case of several of the plots in Figure 17, the fitted curves appear to be parabolic rather than the expected sigmoidal shape. The parabolic shape is due to the curve fitting software determining that the inflection point in the dose response curve rests at a concentration outside the range of data points available within the plot. For this screening assay, the conjugates were tested near their solubility limit and therefore higher concentrations of conjugate could not be tested in order to complete the dose response curves and produce the typical sigmoidal plots.
Conjugate #25
Dose Response Curve Fit

Conjugate #26
Dose Response Curve Fit

Conjugate #38
Dose Response Curve Fit

Conjugate #39
Dose Response Curve Fit
Figure 17: TF-1 cell proliferation assay quantifying conjugate response. 25,000 TF-1 cells/well. Graphs depict the normalized resazurin response of conjugates #25, 26, 38, 39, 41, 42, 51, 54, and 57. Y-axis normalized to the positive control, EPO (10 Units/mL), and negative control being untreated TF-1 cells. DMSO concentration of 0.5% v/v. The curve fit for conjugates #25 ($R^2=0.99$), #26 ($R^2=0.99$), #38 ($R^2=0.97$), #41 ($R^2=0.99$), #42 ($R^2=0.99$), #54 ($R^2=0.97$), #57 ($R^2=0.99$) are significantly better than the fit of the function $y=\text{constant}$. The curve fit for conjugates #39 ($R^2=0.93$), #51 ($R^2=0.94$) are not significantly better than the fit of the function $y=\text{constant}$. $n=3$
Further testing of the nine conjugates of interest determined that only conjugates #25 and 27 were bioactive (Figure 18). These two conjugates were the only samples in which an ANOVA analysis revealed that their fluorescence values were significantly different than the negative control. Still at several concentrations they did not exhibit any significant difference from the negative control despite low standard deviation values. The highest level of bioactivity these conjugates display is approximately 30% of the activity of EPO (Figure 18). In comparison, the monomeric EMP1 peptide exhibits approximately 40% of the bioactivity of EPO at its peak.

Figure 18: TF-1 cell proliferation assay comparing the activity of EPO to conjugates #25 and #27. 25,000 cells/well. Y-axis normalized to the positive control, EPO (10 Units/mL), and negative control being untreated TF-1 cells. DMSO concentration of 0.0 5% v/v. Concentrations of conjugate that show statistical significance (p<0.05) are denoted with a * with corresponding color. n=3.
3.4 QK Conjugates

The QK conjugates were synthesized at the same concentrations as the EMP1 conjugates, however, they did display higher solubility than the EMP1 conjugates and therefore required a lower dilution factor prior to addition to media. The QK conjugates were diluted in DMSO to 1/16 of their initial concentration prior to use in assays, making the max concentration used among the conjugates approximately 1.25µM. The assay to screen the QK conjugates for potential bioactivity contained 0.5% DMSO due to the conjugates being suspended in DMSO after synthesis and addition to culture media at 1% v/v. While this DMSO concentration did not affect the TF-1 cells significantly, it results in a marked reduction in HUVEC viability. Due to this factor, a control plate of VEGF and QK dilutions was run in the presence of 0.5% v/v DMSO in addition to a control plate containing no DMSO. Having these controls allows us to determine how much of an effect DMSO is having on the cells in the assay, as well as if a response to VEGF and QK can still be detected at this DMSO concentration. For the proliferation assay, it was determined that the DMSO concentration yielded approximately a 25% reduction in cellular viability as compared to the control plate containing no DMSO (Figure 19).
3.4.1 Effect of QK Conjugates on Cellular Proliferation

Like the EMP1 conjugates, many of the QK conjugates did not exhibit any response above the negative control. A subset of conjugates did generate a response from the HUVEC. Due to the high standard deviations and the effect of DMSO, conjugates that resulted in at least one concentration that displayed a significant difference from the negative control were included for further testing (Figure 20). These conjugates included numbers 17, 23, 28, 31, 32, 33, 34, 35, 39, 44, 45, and 50.
Figure 20: Response to QK conjugates of interest. HUVEC proliferation assay, 10,000 cells/well in 96-well plates. Graphs depict the normalized resazurin response of conjugates # 17, 23, 28, 31, 32, 33, 34, 35, 39, 44, 45, and 50. Y-axis normalized to the positive control, VEGF (25 ng/mL) and 0.5% v/v DMSO, and negative control being untreated HUVEC exposed to 0.5% v/v DMSO. n=3
The conjugates of interest depicted in Figure 20 were tested again with reduced DMSO concentration, which should provide a better picture of the bioactivity these conjugates exhibit. Upon further testing the data revealed that none of the selected conjugates induced a cellular response that was significantly different than untreated HUVEC. There were two conjugates, #23 and #44, that reproducibly had average fluorescence values above the negative control. However, due to the standard deviations of these values, they are not significantly different from the negative control (Figure 21). Performing an ANOVA analysis on the fluorescence values obtained for these two conjugates confirmed that their response is not significantly different from untreated cells.

![Figure 21: HUVEC proliferation assay. Comparison of VEGF response to QK conjugates #44 and #45. Conjugate activity is not significantly different than the negative control ($p > 0.05$). Conjugates were tested in media containing 0.05% DMSO. $n=3$.](image)

After screening of the conjugates through the proliferation assay, the next step was to test any conjugates exhibiting some level of bioactivity against other cellular pathways.
to determine the full scope of the conjugate’s mimetic ability. While QK conjugates #23 and #44 did not exhibit significant bioactivity, they display the highest average fluorescence values out of all 60 conjugates tested.

3.4.2 Effect of QK Conjugates on Cellular Migration

An in vitro wound healing model (scratch test) was used to test the effect conjugates had on cellular migration. For this assay, HUVEC were grown to confluency in 24-well plates. The confluent monolayers were then scratched with p200 pipette tips and cellular invasion back into the “wound” area was quantified. Migration was quantified by measuring wound area at time, t = 0 hours, and comparing it to wound area measured at time, t = 18 hours (Figure 22). When preforming a scratch assay, it is important that all wounds have approximately the same widths as the rate of wound closure is indirectly proportional to wound size. Therefore, if one sample has a smaller initial wound size, it may appear that a sample is causing the generated wound to heal more quickly when in fact the increased wound closure is due to the reduced wound size. When creating the scratches, the same size pipette tip must be used for all samples and care must be taken that the pipette tip is flat against the surface of the well to produce wounds of the same width in all wells. The field of view for images taken at t= 0 hours and t= 18 hours were matched as closely as possible which was simplified by marking the underside of the well plates with a marker and lining up the marking for each picture. Analysis of all wound healing images was preformed using the ImageJ software.
**Figure 22:** HUVEC migration assay. Evaluation of migration due to conjugates of interest, #23 and #44.

Scratches in cell monolayers made with p200 pipette tip.
Table 3: Statistics for HUVEC migration assay. All samples significantly different from negative control, untreated cells \(p<0.05\), n=3.

All samples and controls exhibited wound closures that were significantly different than the negative control as confirmed by ANOVA analysis (Table 3). It has been reported that QK exhibits higher wound closure than VEGF, however in this assay QK reproducibly exhibited significantly lower wound closure than VEGF [28]. The results of the assay indicated that conjugates #23 and #44 are capable of enhancing migration in HUVEC. The conjugates produced a response comparable to the monomeric QK peptide, but that were significantly different from the response to VEGF.

3.4.3 Effect of QK Conjugates on Tubule Formation

As vascular cells, HUVEC and other endothelial cells exposed to angiogenic factors, are able to form capillary-like tubule structures. Potential VEGF mimetic conjugates must be able to activate the cellular pathways involved in angiogenesis and result the formation of tubules. To perform this assay, HUVEC require a material that they form strong focal adhesions with, such as basement membrane extracts (BME) like Matrigel. Endothelial cells do not form these structures well when plated on gelatin coated plates, which is adequate for a proliferation assay. Matrigel has a high degree of angiogenic activity on its own as HUVEC grown on Matrigel coated surfaces will undergo tubule formation due to the presence of cytokines and growth factors present in the
membrane extract. Normal Matrigel is useful for anti-angiogenic experiments, but for testing of pro-angiogenic polymer-peptide conjugates, a growth factor reduced (GFR) Matrigel must be used to reduce the non-VEGF dependent angiogenic response from HUVEC.

**Figure 23:** HUVEC angiogenesis assay. Quantification of tubule structures on GFR Matrigel coated 24-well plates. Images taken 16 hours after cell plating.
Sample | Total Tubule Length (mm) | Standard Deviation (mm)
---|---|---
VEGF | 23.21 | 1.84
QK | 23.75 | 2.28
Untreated Cells | 17.59 | 0.28
Conjugate #23 | 26.18 | 2.41
Conjugate #44 | 1.48 | 0.37

Table 4: Statistics for HUVEC angiogenesis assay. All samples yielded total tubule lengths that were significantly different ($p<0.05$) from that of the negative control, untreated cells. n=3.

The images collected for this assay (Figure 23) were analyzed using the ImageJ software, making use of an automated angiogenesis analyzer available for download from the macros section of imagej.nih.gov. There are many ways to quantify the angiogenic response displayed in this type of assay, number of tubule branches, number of tubule node/junctions, number of complete circles formed by tubules, and total tubule length are all metrics that can be quantified [4,25,34,38]. As we are making use of an automated analyzer, total tubule length can be easily obtained and is the metric selected for use.

Results show that all samples tested, including the negative control, exhibited some level of tubule formation, except for conjugate #44, which appears to have inhibited tubule formation (Table 4). All controls and samples showed significance as compared to the negative control after preforming an ANOVA analysis on the data. Interestingly, there is also no significant difference between VEGF, the QK peptide, and conjugate #23, with each inducing a similar amount of tubule formation.
4: Conclusions

The goal of this work was to develop a set of polymer-peptide conjugates to test and determine if they are able to retain or enhance the bioactivity of the monomeric peptide. The polymer-peptide conjugates are multivalent, having several peptide binding units, however, that does not mean that they present those binding units to receptors. The conjugates may undergo folding that results in internalization of peptide units, or they may fold in such a way that they do not present peptide units in an appropriate orientation to bind and subsequently dimerize and activate their receptors. The capacity to form large polymer libraries, utilizing oxygen tolerant RAFT polymerization, is relatively new and therefore there is not much data on polymer folding, which makes it difficult to predict which combinations of monomers will yield a successful protein mimetic conjugate. Developing a large set of conjugates serves to increase the likelihood of finding a potential mimetic conjugate.

There are only a few cases for how a polymer backbone can fold and display peptide units: 1) the backbone can internalize the peptide units, not presenting them for binding with a receptor, 2) the backbone can remain in a linear chain, presenting peptide units along its length, 3) or the polymer backbone can fold and present peptide units close together. An unfolded linear conjugate would likely result in bioactivity similar to a monomeric peptide due to spacing between peptide binding units, although it may be the case that this peptide spacing is optimal for the receptors and enhances bioactivity. A folded conjugate that presents its peptides close together should benefit by being able to present multiple binding units within the binding pocket formed by dimerized receptors, however, this interaction is unknown.
From the data collected there appears to be no conjugate that stands out, exhibiting enhanced bioactivity. Instead the data suggests that there are at least two EMP1 conjugates, #25 and #27, and two QK conjugates, #23 and #44, that retain some level of bioactivity. The EMP1 peptide is hydrophobic, which is why the EMP1 conjugates had lower solubility and had to be tested at lower concentrations than the QK conjugates. This hydrophobicity may also mean that it is more likely that the EMP1 conjugates fold and internalize their peptide units. This is particularly true for very hydrophilic polymer backbones, such as ones with high incorporations of PEG based monomers.

The EMP1 conjugate #25 appears to have two peaks in its activity. The reason for this is unknown but may be a result of conjugate-conjugate interactions and aggregation. Conjugates #25 and #27 did not exhibit a significant difference from the negative control at several of the higher concentrations tested which again may be due to conjugate-conjugate interactions. The peak activity seen for both of these conjugates is lower than that of monomeric EMP1, suggesting that they do not benefit from multivalency. Both of these conjugates have polymer backbones only consisting of only HEA and NHS monomers, conjugate #25 containing 5% NHS incorporation, and conjugate #27 containing a 10% NHS incorporation. The fact that both conjugates are HEA homopolymers may indicate that the difference in hydrophobicity/hydrophilicity between the backbone and peptide units is close enough to not result in internalization of the peptides, although it is not possible to determine if the conjugates form a linear chain or not. Interestingly, conjugate #26 did not show any activity after further testing with reduced DMSO, despite the fact that it is also an HEA homopolymer containing 7.5% NHS incorporation, a median between conjugates #25 and #27. Conjugate #57, which was of interest in the initial
proliferation screening, is a homopolymer of 2HPMA that did not result in significant bioactivity. This may indicate that HEA is significant in display of bioactivity seen among this set of conjugates.

For QK conjugates #23 and #44, neither exhibited activity that was significantly different from the negative control in the proliferation assay with reduced DMSO. However, they were the only two with reproducibly higher average resazurin fluorescence values for that assay. After further testing of these two conjugates in the migration assay, it was observed that both displayed induction of cellular migration on the level of the QK peptide itself, but still significantly lower as compared to VEGF. The results of the angiogenesis assay contradict the findings of the migration assay, with QK conjugate #44 seeming to inhibit the formation of tubules. The reason for this is unknown, however, may indicate that while the conjugate is capable of binding to VEGFRs, as seen by induction of migration, it does so in a way that diminishes receptor dimerization. Conjugate #23 on the other hand displays the same level of angiogenesis induction as VEGF and QK. The polymer backbone of conjugate #23 consists of equal parts HEA and mPEG300 with 10% BA and 7.5% incorporation of NHS. The backbone of conjugate #44 consists of equal parts 2HPMA and PEGMA with 5% MMA and 7.5% incorporation of NHS. These conjugates both have very hydrophilic backbones, with the same percent NHS incorporation, which may indicate similar peptide spacing and orientation within the conjugates. This orientation may be the reason they stood out in the proliferation assay, and also the reason neither exhibit enhanced bioactivity. One of the conjugates of interest after the initial proliferation screening assay, conjugate #28, consisted of 95% mPEG300 and 5% NHS. Being a homopolymer of mPEG300, the conjugate likely forms a linear chain, which indicates that
the inclusion of HEA is significant to the activity conjugate #23 displays, potentially resulting in folding of the polymer backbone. Taking the results of the proliferation, migration, and angiogenesis assays together, it can be concluded that neither conjugate #23 or #44 benefit from multivalent peptide display.

Further investigation into the use of polymer-peptide conjugates as potential mimetics must be done, including expansion of the polymer library used to develop the conjugates used in this study. We have shown that conjugates can retain the activity of monomeric peptides and although this set of conjugates did not exhibit enhanced bioactivity, there are many more possible combinations of backbones and additional monomer types yet to be tested. Combinations that may result in proper folding and display of peptide binding units to receptors.

5: Future Directions

This study consisted of only 60 polymer-peptide conjugates, all with DP 200. There are many ways in which the set of conjugates could be expanded on. DP, monomer composition, and peptide valency are all parameters that can be easily modified and investigated further. Additionally, the polymers used were all randomly distributed heteropolymers of different monomer units, however, block copolymers could be used as well. Block copolymers are polymers in which the various monomer components are separated into segments within the polymer chain, which may result in vastly different conjugate folding as compared to a heteropolymer with the same ratio of monomer components. It is also possible to synthesize polymers with different shapes, the conjugates used in this study were all linear chains, but 3-arm and 4-arm polymers could be included.
It is important to characterize the conjugates at various stages during synthesis. Nuclear magnetic resonance (NMR) can be utilized to determine the success of polymerization. GPC and MALS data allow us to determine success of polymerization by measuring molecular weight, however these methods can have issues with reliability and reproducibility [39]. NMR does not rely on calibration standards like GPC data and is considered more reliable [39]. NMR allows for the determination of the concentrations of molecular species present in a solution by measuring the intensity of characteristic hydrogen peaks of those species [39]. For our purposes, the success of a polymerization reaction can be determined by measuring the shifts in intensity of characteristic hydrogen peaks associated with monomer units. As monomers are consumed in a reaction, the peaks should decrease in intensity.

Various additional characterization methods can be employed prior to biological testing, such as surface plasmon resonance (SPR). Receptors can be immobilized onto SPR sensors and used to measure the capacity for samples to bind those receptors [40]. This data would provide insight into the binding affinity conjugates have for the cellular receptors of interest [40]. SPR data would complement biological data revealing if bioactivity is due to receptor binding and activation or if it is due to another unknown interaction between cells and conjugates.

Future work should also seek to include more robust controls throughout experiments. Controls that could be added include conjugates containing no NHS, as well as conjugates containing scrambled versions of the EMP1 and QK peptides. Use of such controls would serve to eliminate the possibility that any bioactivity exhibited by conjugate samples may be due to sources other than receptor clustering and activation.
Currently, it is not possible to predict the folding of synthesized conjugates and the orientation of their receptor binding units. Being able to predict these characteristics would be invaluable in the design of our conjugates. Informed design would allow us to produce conjugates that are the most likely to present peptides in an optimal orientation to receptors. Techniques such as small-angle X-ray scattering (SAXS) and small-angle neutron scattering (SANS) allow for the determination of conjugate size, compactness, flexibility, and peptide display [41]. These techniques measure the radial structure of molecules, which in this case can provide information on conjugate compactness [41]. A conjugate with a high ratio of radius of gyration to hydrodynamic radius can be expected to be similar to a disordered linear chain, whereas conjugates with a lower radius of gyration have undergone some degree of compaction and are more ordered [41]. Deuterium labelling would allow for the differentiation between polymer backbone and peptide within a conjugate due to their characteristic scattering length densities and therefore provide insight into how the conjugate is, or is not, presenting peptide units [41]. Our polymer-peptide conjugates are meant to mimic proteins which form very ordered structures, undergoing high degrees of folding, ideally the conjugates would exhibit some level of order as well, while presenting peptide binding units in a configuration that enhances receptor clustering. The use of small angle scattering techniques would provide information that could lead to the ability to design conjugates with ideal characteristics.

Future studies will also investigate the co-functionalization of polymer backbones with peptide QK and an RGD peptide for potential enhancement of cellular response. RGD peptides as the αβ3 integrin has been linked to activation of VEGF receptors. RGD peptides are ligands for αβ3 and αβ5 integrins [42]. The role of cellular adhesion in
VEGF signaling has been explored, revealing that there is an interaction between the αvβ3 integrin and VEGFR-2 that enhances angiogenesis [42]. The co-stimulation of both VEGFRs and αvβ3 could further potentiate VEGF signaling.

EPO and VEGF activate closely related pathways in their target cells, such as proliferative and cell protective pathways. Interestingly, it has been found that due to a shared precursor cell type, known as a hemangioblast, between erythroid and endothelial cell lineages, hematopoietic stem cells are capable of acting as endothelial progenitors and can localize to sites of angiogenesis [43]. These hematopoietic stem cells have receptors and can be stimulated by both EPO and VEGF [43,44]. While binding of EPO to EPOR dimers results in erythropoiesis, it was found that binding of EPO to a heterodimer of EPOR and common β receptor (βC-R) results in activation of the eNOS pathway [8, 43,44,45]. The cell protective effect of EPO comes from stimulation of EPOR/βC-R heterodimers [43]. Nitric oxide production in endothelial progenitor cells (EPC) leads to cell maturity, it regulates proliferation, apoptosis, and differentiation in these cells [43,44]. Nitric oxide therefore plays a significant role in cell recruitment during wound healing [43]. In addition to stimulation by these two growth factors, it has been established that colocalization of EPOR/βC-R and VEGFR-2 is required for induction of eNOS in EPC [43,44]. The combined action of these receptors has implications for the use of our EMP1 and QK conjugates, as future studies can investigate co-functionalization of these mimetic peptides to target both VEGFR and EPOR/βC-R dimers to improve wound healing over VEGF stimulation alone. Although the use of EMP1 in this case will also need to be investigated, as the induction of erythropoietic signaling by EPOR homodimers can result in the interaction of thrombocytes with vascular endothelium leading to thrombosis [46].
If it is found that EMP1 is not suitable for this application, peptides such as ARA290 have been developed that are based off of the binding domain of EPO, but have been found to be nonerythropoietic, meaning that they selectively bind EPOR/βC-R heterodimers [45,46].
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