Experimental Estimate of the Diffusivity of Vascular Endothelial Growth Factor
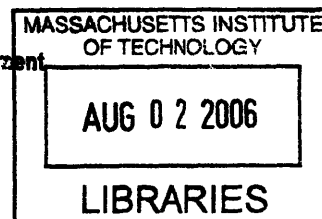
by

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EXPERIMENTAL ESTIMATE OF THE DIFFUSIVITY OF VASCULAR ENDOTHELIAL GROWTH FACTOR

By

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ABSTRACT

The diffusivity of Vascular Endothelial Growth Factor (VEGF) is a number that is very important in determining the transport of VEGF. The transport of VEGF determines crucial processes such as angiogenesis and vasculogenesis. This study aimed at obtaining an estimate of the diffusivity of VEGF by using a simple system consisting of an insert with a collagen membrane placed within the well of a cell culture plate. A solution of VEGF was added to the insert, and the same solution without VEGF was added to the well surrounding the insert. The VEGF diffusion was monitored by taking samples in time of both the upstream and downstream baths, and analyzing the samples with an ELISA. Modeling the diffusion as one-dimensional, it was possible to estimate the diffusivity from the change in downstream concentration with time. The diffusivity was estimated to be around $5.55e^{-7}$ cm^2/sec.

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1.0 INTRODUCTION

The transport of vascular endothelial growth factor (VEGF) is a very important event in determining the morphogenesis of blood vessels. Angiogenesis is the branching of blood vessels from pre-existing blood vessels. Vasculogenesis is the formation of new blood vessels. These are the two processes by which blood vessel morphogenesis takes place. Both of these processes begin when VEGF binds to a receptor on the surface of endothelial cells. This binding begins a kinase-based signaling cascade that serves to inform the cell to migrate in the direction of the sensed VEGF gradient. For VEGF, as with other morphogens, the local gradient relative to the migrating cell, rather than the absolute concentration of VEGF, is what drives migration.

The formation of VEGF gradients is governed by the transport of VEGF. The transport of VEGF, in turn, is governed mostly by diffusion and fluid convection. The gradient of VEGF will be determined by the balance of these two forces. One number that is critical in determining the transport of VEGF within the interstitial space is the diffusivity of VEGF. The diffusivity of VEGF is what, together with the concentration gradient, determines its rate of diffusion.

Theoretical approximations for the diffusivity of VEGF have been quoted in the literature. Helm et al. assume that VEGF is a dimmer of 64 kDa, and assume that its diffusivity is $7e^{-7}$ cm^2/sec.[1] Their experiment required the use of a bi-domain fusion protein comprised of mature recombinant $VEGF_{121}$ and a domain that can bind fibrin, which explains the high molecular weight. Another estimate comes from Gabhann and Popel.[2] They estimated a diffusivity of $1.05e^{-6}$ for $VEGF_{121}$, and a diffusivity of $0.94e^{-6}$ for $VEGF_{165}$ at 23°C. Gabhann and Popel also work with a range in between $10e^{-5}$ to $10e^{-7}$ for all VEGF in another study.[3]

VEGF has various isoforms ranging from 35-44 kDa. These include $VEGF_{121}$, $VEGF_{165}$, $VEGF_{189}$, $VEGF_{206}$, VEGF-B, VEGF-C, VEGF-D, VEGF-E, PIGF-I, and PIGF-II. The Einstein relation of kinetic theory and Stoke's Law predict that the Diffusivity of any given spherical molecule in low Reynolds number flow is inversely proportional to the first power of the particle's radius.

$$D = \frac{k \cdot T}{6 \cdot \pi \cdot \eta \cdot r} \tag{1}$$

Here r is the particle's radius, k is Boltzmann's constant, T is the absolute temperature, and η is the viscosity of the medium. This relation can be used to estimate the diffusivity of globular proteins if r is taken to be the effective radius of the protein. Using this relation, and assuming that a heavier protein also has a proportionately larger radius, it can be predicted that the VEGF isoforms with the highest molecular weights have the lowest diffusivities.

The goal of this study is to experimentally determine the diffusivity of $VEGF_{121}$ in collagen. This number can then be used to estimate the diffusivities of other isoforms of VEGF in free space and in other substances.

2.0 THEORY

2.1 Theory Behind the Model for the Experiment

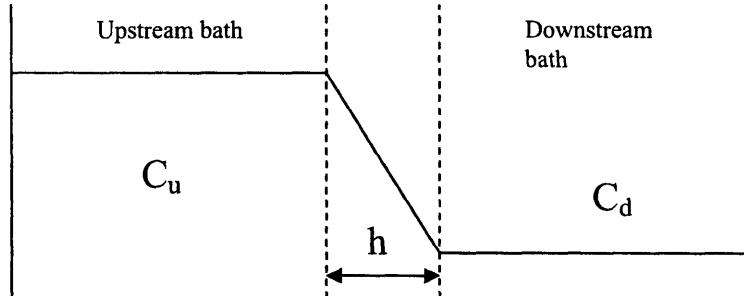Figure 1 depicts a model for the experimental apparatus used.

Figure 1: Model of Diffusion across the membrane. The diffusion experiment can be modeled as two well-mixed chambers separated by a membrane of thickness h. The upstream bath has some concentration C_u, and the downstream bath has some lower concentration C_d. After a long enough time period, the concentration gradient across the membrane can be modeled as linear.

The situation can be modeled as a chamber consisting of two well-mixed baths separated by a membrane of a given thickness. One of the baths, the upstream bath will have some initial concentration of VEGF, and the downstream bath will have no VEGF. For this model the one dimensional version of the diffusion equation will be used.

$$\phi = -D\frac{dC}{dx} \tag{2}$$

Here φ is the flux, C is the concentration, D is the diffusivity, and x is the direction along the membrane, which is taken to be positive in going from the upstream bath to the downstream bath. The diffusion can be modeled as one dimensional because the thickness of the membrane is thin compared to area of the membrane.

The concentration profile across the membrane will initially be non-linear, but after a long enough period of time, the profile becomes linear. This progression is depicted in the following figure.
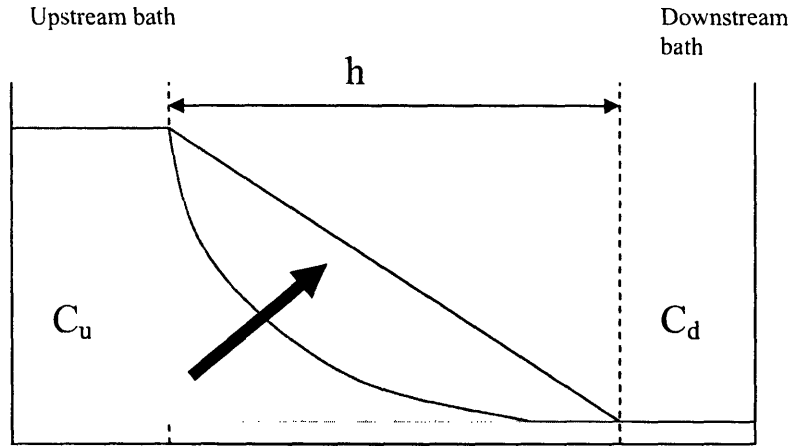
Figure 2: Linearization of the concentration profile within the membrane. The concentration profile within the membrane will at first be zero. It will then quickly start rising from the upstream end. After enough time, the profile will become linear. At zero time, the profile looks like the green curve. After some time it progresses to look more like the red curve, until finally it becomes linear, like the blue curve.

The profile at t=0 looks like the curve at the tail of the arrow. With time the concentration profile progresses and becomes linear, like the curve at the tip of the arrow above. The amount of time required for the profile to become linear given by the following equation.

$$\tau_{linear} = \frac{h^2}{\pi^2 \cdot D} \tag{3}$$

In this equation τ_{linear} is the time constant to linearity, and h is the thickness of the membrane.

After the concentration profile has become linear, the change in concentration across the membrane is given by,

$$\frac{dC}{dx} = \frac{C_d - C_u}{h} \tag{4}$$

Where C_d is the uniform concentration of the well-mixed downstream bath, and C_u is the uniform concentration of the well-mixed upstream bath. Combining equations 1 and 3 gives the following equation,

$$\phi = D\frac{C_u - C_d}{h} \tag{5}$$

Another equation for flux can be obtained from the change in downstream concentration in time when the flux is constant.

4

$$\phi = \frac{V_d}{A} \cdot \frac{\Delta C_d}{\Delta t} \tag{6}$$

In this equation, V_d is the volume of the downstream bath, ΔC_d is the change in concentration of the downstream bath, A is the area of the membrane available for diffusion, and Δt is the time elapsed.

Equations 4 and 5 can be combined to give the diffusivity.

$$D = \frac{C_u - C_d}{h} \cdot \frac{V_d}{A} \cdot \frac{\Delta C_d}{\Delta t} \tag{7}$$

Here the flux is assumed to be both linear and constant. The assumption of linearity is valid as long as the time elapsed is greater than τ_{linear}. In order for the flux to be constant the concentration difference in between the upstream and the downstream bath also has to remain constant. If the downstream concentration is negligible when compared to the initial upstream concentration, and the upstream concentration remains relatively constant, the flux can be assumed to be constant. This simplification reduces equation 6 to the following.

$$D = \frac{C_u}{h} \cdot \frac{V_d}{A} \cdot \frac{\Delta C_d}{\Delta t} \tag{8}$$

Using this equation, the diffusivity is easily calculated from the slope the downstream concentration curve as a function of time.

2.2 Calculation of the Diffusivity of VEGF

Equation 7 above was the equation used to estimate the diffusivity of VEGF. The downstream volume, the area available for diffusion, and the thickness of the collagen gel were calculated from the dimensional parameters of the insert, the dimensional parameters of the well, and the volumes used in the experiment. The insert had an inner diameter of 1cm and an outer diameter of 1.2cm. The height of the insert was 1cm. This includes three feet at the base of the insert that are 1mm to 2mm tall. The well had a diameter of 2.21cm. The area available for diffusion was calculated from the inner diameter of the insert.

$$A = \pi \cdot \left(\frac{D_{in}}{2} \right)^2 \tag{9}$$

In this equation, D_{in} is the inner diameter of the insert. The thickness of the collagen gel was calculated to be the volume of collagen placed in each insert, divided by the area available for diffusion.

5

$$h = \frac{V_{col}}{A} \qquad (10)$$

Here V_{col} is the volume of collagen that was used for each insert, which in this experiment was 200 microliters. The volume of the downstream bath was calculated to be the volume of the well minus the volume taken up by the insert.

$$V_d = \pi \cdot \left(\frac{D_w}{2}\right)^2 \cdot \left(h + \frac{V_u}{A} + 0.1\right) - \pi \cdot \left(\frac{D_{out}}{2}\right)^2 \cdot \left(h + \frac{V_u}{A}\right) \qquad (11)$$

In this equation D_w is the diameter of the well, V_u is the upstream volume, which in this study was 500 microliters, and D_{out} is the external diameter of the insert. The value of 0.1 used in the equation is added to represent the height of the feet of the insert. The reason for using 1mm for the height of the feet instead of 1.5 or 2mm is that the height of the fluid in the well in the experiments did not exactly reach the height of the fluid in the well.

The upstream bath concentration and the concentration of the downstream bath with time were both obtained from the concentration values obtained from the samples taken during the course of this experiment. For the downstream bath, the slope of the plot of concentration as a function of time was taken from a linear fit to the data. For the upstream concentration, the values plotted in the graph of upstream concentration with respect to time were averaged for each insert, excluding obvious outliers, and the resulting average was used in calculating the diffusivities. Only the values after 5.5 hours where used in obtaining these average upstream concentrations.

The results section contains the actual values that were used in calculating the diffusivities for each insert from equation 7.

3.0 APPARATUS AND EXPERIMENTAL DESIGN

3.1 Apparatus

In order to simplistically approximate the situation modeled in the theory section above the apparatus used consisted of a Millipore 12mm cell culture insert, with a collagen gel membrane, inserted into one of the wells of a corning 12-well tissue culture plate. Three inserts were used in one plate. The situation is diagramed below.
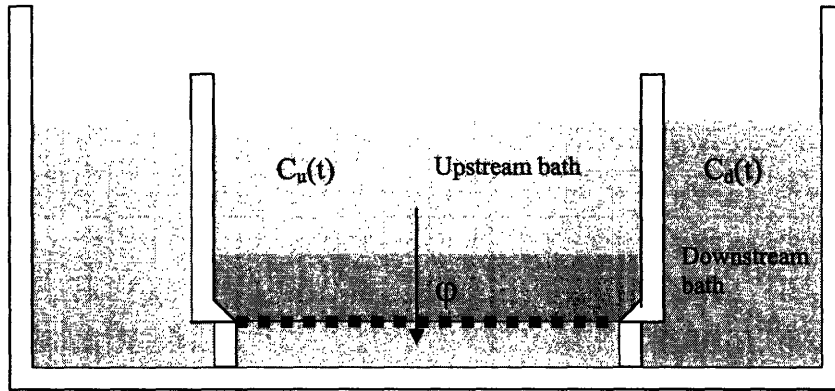
Figure 4: Apparatus Diagram. An insert, filled with collagen, was placed in one of the wells of a 12-well tissue culture plate. A solution with VEGF was pipetted into the insert, while that same solution without VEGF was pipetted into the well.

The solution inside of the insert contained VEGF, while the solution outside the insert did not. The flux of VEGF occurred from inside the insert to outside the insert.

3.2 Procedure

3.2.1 Collagen Preparation

Collagen was prepared from a concentration of 4.27 mg/ml to a concentration of 2 mg/ml by diluting in 1X PBS and equilibrating with NaOH. The gels were prepared in the inserts used in the experiment. The gels were incubated for half an hour, after which the solution with VEGF was pipetted into each insert.

3.2.2 Experiment

After the collagen gels were incubated for half an hour, 500 microliters of a solution of 200 ng/ml of human VEGF and 0.1% BSA in 1X PBS was added to each of three inserts. The wells in which the inserts were placed were filled with a solution of 0.1% BSA to a height slightly under the height of the fluid within the insert. The 12-well plate with the three inserts was then placed in the incubator. After half an hour, the plate was taken out. Fifty microliters were taken from each insert and replaced with 50 microliters of a solution of 200 ng/ml VEGF and 0.1% BSA in 1X PBS. The downstream bath for each well was mixed. The sampled 50 microliters were then diluted to a concentration of 5 ng/ml, and the plate was placed back into the incubator. This was repeated every half an hour up to five hours from when the inserts where first filled. After the third hour the VEGF source was changed.

After five and a half hours from when the insert was first filled, samples from the downstream bath, the well, were taken along with the samples from the upstream well. This was done in order to only take samples when the concentration profile was expected to be linear. From five and a half hours to eight hours, the gels were taken out of the incubator. Fifty

microliters were taken from the insert and diluted. Another fifty microliters of the 200ng/ml VEGF solution were added to the insert. The well was mixed, and 40 microliters were taken from the well. The plates were then returned to the incubator. These steps were repeated for each insert for each of the half hour intervals.

3.2.3 VEFG Detection

The VEGF concentration of each sample taken was analyzed with an ELISA assay from PeproTech Inc. The protocol for this assay comes with the kit, and is found in their website. The protocol was followed almost exactly as specified. The only difference was that the washing solution in this experiment also contained 0.1% BSA. Another difference in between the protocol and the steps followed in this study is that in washing, the plates were not aspirated. Instead, a squeeze bottle with the wash solution was used. This was done in order to avoid scraping the plates, which might lead to removal of the blocker, and non-specific binding of the detection antibody or the peroxidase to the walls of the well. Non-specific binding leads to a false positive signal. BSA was used in every step of the ELISA protocol, including the washing, in order to reduce non-specific binding.

The samples from the experiment were iced before being assayed. The washing solution was left at room temperature for about a day. The avidin peroxidase had not been aliquoted and frozen immediately upon arrival at the lab.

Sample concentrations of VEGF were prepared in order to assay with the samples from the experiment. Because the concentration of the samples is known, obtaining optical density values for these allows for the calculation of concentration values for the samples from the experiment based on their optical density values.

The ELISA plate contained 96 wells total. The samples from the top of the inserts, 16 per insert, each was assayed once, in one well. Thirty wells were dedicated to the sample concentrations of VEGF. The remaining 18 wells were used to assay the 6 downstream samples for each insert.

4.0 RESULTS

The standard curve is the plot of optical density as a function of VEGF concentration. This plot was obtained as explained above from samples of known concentrations.
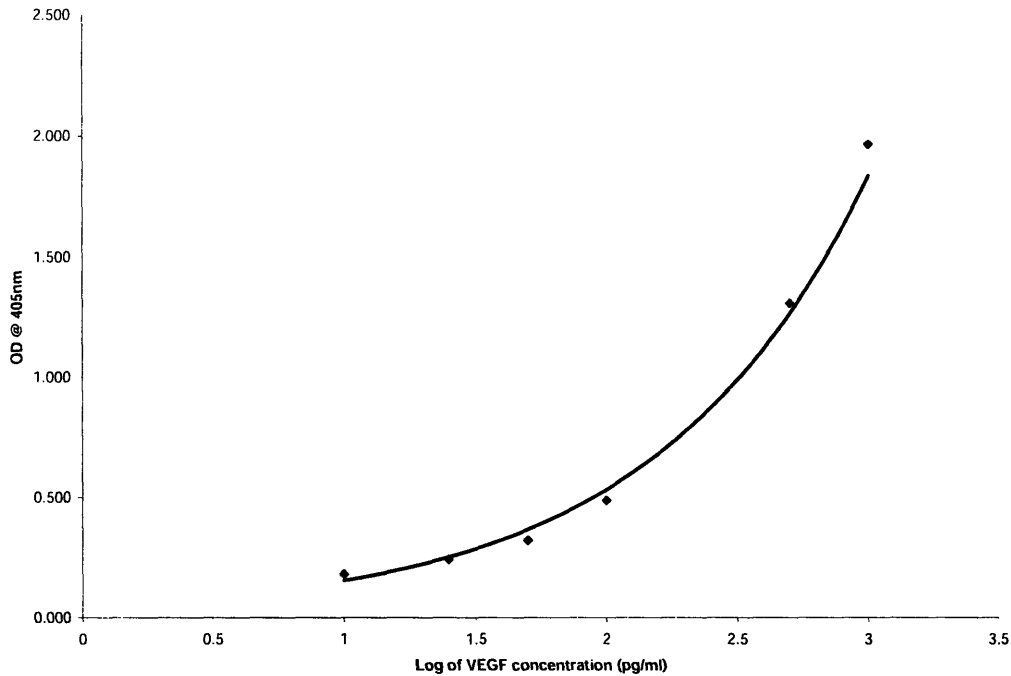
Figure 5: Standard Curve. Optical density is plotted as a function of concentration. The fit to this curve is exponential and is used to assign a concentration to each optical density value obtained in this experiment.

The fit in this plot is an exponential. The equation for this fit was used to assign a concentration to each optical density value obtained from the ELISA assay.

The samples taken from the downstream bath (the well) were assayed and the optical density was surveyed three times. The surveying of the plate was done as quickly as possible to attempt to get multiple readings of the same optical density. The plate was surveyed three times within two minutes. These values were averaged for each insert and they were converted to concentration values by using the formula obtained for the standard curve. These concentration values were adjusted for their original dilution, and plotted here.
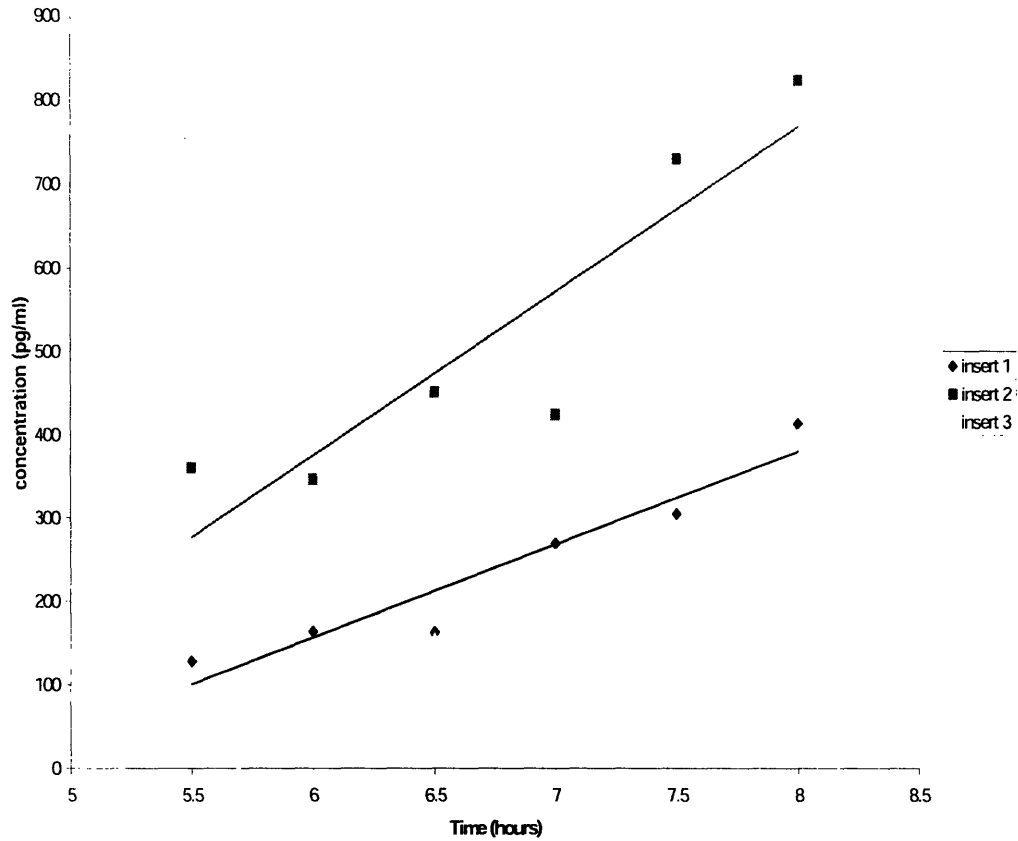
9

Figure 6: Concentration vs. time for the downstream compartment. The downstream concentration for each insert is plotted as a function of time. These concentration values are obtained from the optical density obtained for each downstream sample.

The upstream concentration optical density values were also surveyed three times within a short time span, and averaged. The averaged optical values were then converted to concentration values using the standard curve. The results are plotted here.
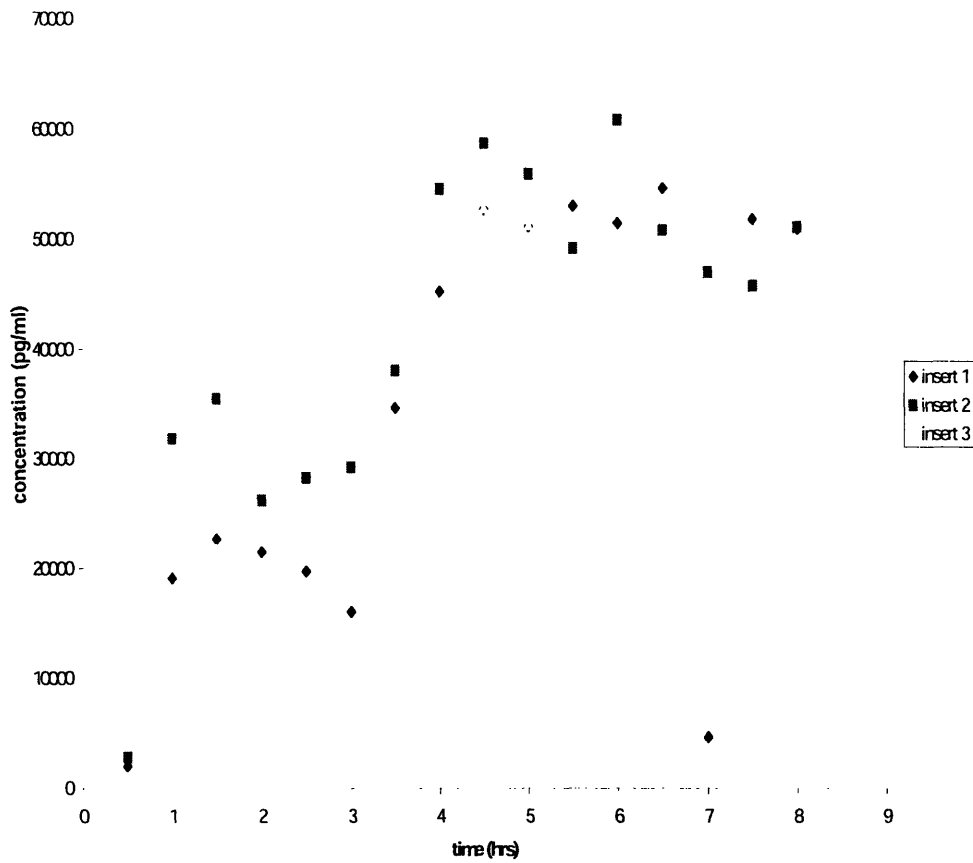
Figure 7: Upstream concentration as a function of time. Optical Density values for the upstream concentration were converted to concentration values and plotted for each of the inserts as functions of time.

The values for upstream concentration plotted above are also adjusted for dilution.

The diffusivity values for the three inserts were calculated from the second plateau of the upstream concentration plot, and from the slopes of the downstream concentration plots. They are given in the table below.

Table 1: Experimental diffusivity values.

Insert	Diffusivity (cm^2/sec)
1	5.62101E-07
2	9.8988E-07
3	5.50274E-07

These values were obtained using a membrane width of 2.546mm, an upstream concentration of 50,000 pg/ml, a downstream bath volume of 2.7945 mL, and a membrane surface area of 7.854 mm^2.

5.0 DISCUSSION

In discussing the results from above the first topic of interest is the standard curve. Because all the concentration values are based on this curve, it is very critical to the experiment. The standard curve that is shown on the results section is the curve that relates known concentration of VEGF to its recorded optical density value. This particular curve is only valid for the VEGF used in this experiment. To be more specific, it is valid for only the second type of VEGF used in this experiment. A second source of VEGF was used due to a lack of enough of the first VEGF used. This second VEGF source produced optical density values that were about three times larger those produced by the original source of VEGF. Both curves are reproduced below for ease of comparison.
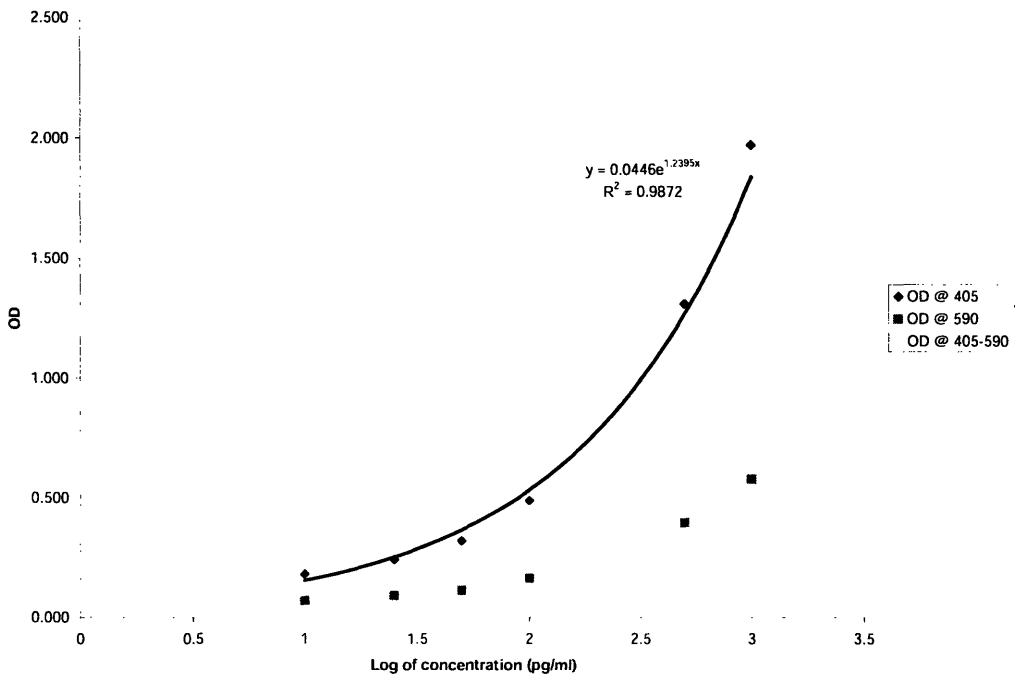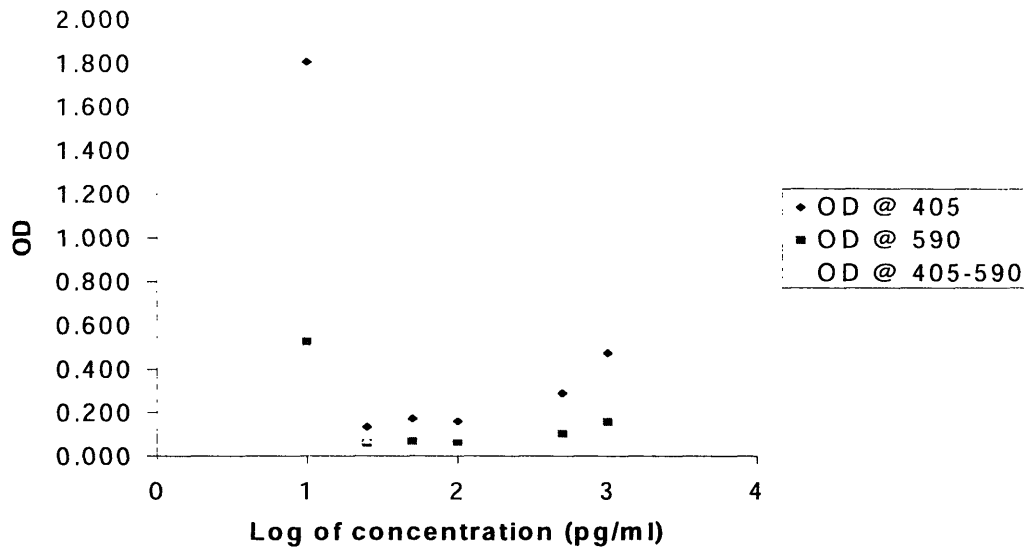
Figure 8: Standard Curves. Standard curves data is plotted for the two different types of VEGF used. The blue diamonds represent the data measured at 405nm. The pink squares represent the data measured at 590nm. The yellow triangles represent the difference in between the optical density values measured at 405 and those measured at 590nm. The standard curve used in this study to obtain the concentration values is plotted here with the equation for the exponential fit and the R value.

While the standard curve looks useful for the second source, the data for the first source does not. This is the case because the lowest concentration gives the highest optical density value, and because the slope of the data after this first concentration value is too small to use in calculating concentration values. The only possible explanation for the high OD value at 10pg/ml for the data pertaining to the first VEGF source is that the 10pg/ml dilution may have been confused for the 10ng/ml solution that was used for making the other dilutions for the standard curve. Even if this is the case, and the OD value at 10pg/ml can be ignored, the slope in the data is not optimal for measuring concentration differences. Because a large increase in concentration produces a small increase in OD, this data would produce a lot of error. For this reason, the standard data for the second VEGF source was used as the standard curve for this study.

The standard curve used, which has an R-value of 0.9872, is a fairly good fit to the standard data. The standard curve closely resembles the curve that is printed in the protocol, which is a good indication that the curve is relatively truthful to the concentration values used.

The procedure for the ELISA calls for the measuring of the OD values to be performed at 405nm corrected to 650nm. Because the plate reader did not have the 650nm setting, this was not possible. Instead, OD values were acquired at 590nm and at 405nm. The idea was to subtract the 590nm readings from the 405nm readings in order to remove any of the OD that is not due to the color of the well, but is instead due to stains on the plate or other obstructions that do not belong to the experiment. However, as can be seen in the standard curves above, the data obtained at 590 does have a pattern. This suggests that the color of the well does affect the OD reading at 590nm, and because it is biased the data at this reading can not be used. For this reason only the values at 405 were used, without the correction.

The upstream concentration is of importance in this study because, as was mentioned in the theory section above, it is proportional to both the flux across the collagen membrane and the diffusivity. The intent of the experimental design was to keep the upstream concentration as constant as possible in order to make the calculating of the diffusivity simpler. From the plot of upstream concentrations with respect to time, it can be seen that this is not the case. This plot, however, has several interesting trends. First, the upstream concentrations at half an hour for each of the inserts is substantially lower than the upstream concentrations at the other time points. The fact that theses concentrations are consistent in both this trend, and in the actual value, suggests that the VEGF was initially up-taken by the collagen at a rate that is much faster than diffusion.

Another point of interest is the two plateaus of the plot of upstream concentrations against time. The first plateau occurs from the end of the first hour to the end of the third hour. After the third hour the upstream concentration rises to a new plateau over the course of an hour and a half. The reason for this observed behavior is that the VEGF source was changed after the third hour. The VEGF that was switched to was the VEGF that gave the highest signal in the ELISA. At any given concentration, this new source of VEGF gave OD values that were about three times as large as the OD values given by the initial VEGF. This translates to a sensed concentration that is about ten times larger for any given concentration. Because of this, when the 50 microliter of the second VEGF replaced the 50 microliter sample of the original VEGF,

the upstream concentration effectively increased. The second plateau is caused by the fact that the upstream VEGF has become most of the VEGF in the insert, and a large amount of it is being diffused across the collagen.

The fact that the upstream concentration remains relatively constant after the fifth hour means that for the calculation of the diffusivity, the upstream concentration can be assumed to remain constant. The upstream concentration does get reduced as time goes on. This is a good indication that the upstream concentration was within the measurement range of the ELISA. The upstream concentration is the result of a fraction of higher-OD VEGF combined with a fraction of lower-OD VEFG. Caution must be taken when calculating the diffusivity. It is not known that the same ratio of the second to the original VEFG is present in both the downstream and the upstream bath.

The diffusivity of VEGF in collagen seems to be around $5.55e^{-7}$. Of the three inserts two gave this value for the diffusivity. The second insert seems to have been more permeable, but because both the third and the first insert are consistent, it is plausible that the value for the diffusivity is closer to this value than to the value obtained from the second insert. Also, it is possible that the second insert was damaged in one of the samplings of the upstream concentration.

6.0 CONCLUSION

The diffusivity of VEGF in collagen is estimated to be around $5.55e^{-7}$. However, because the source of VEGF in this study was switched mid-experiment, it is not absolutely clear that this number is a good estimate. This study does, however, show that a good estimate can be achieved by repeating the experiments with a single and plentiful source of VEGF, and plenty of BSA in every step in order to prevent false positive signal from the ELISA or VEGF denaturalization. Also, in doing the ELISA, the wells should not be aspirated, as specified by the protocol.

SOURCES

1. Helm CL, Fleury ME, Zisch AH, Boschetti F, Swartz MA. Synergy between interstitial flow   and VEGF directs capillary morphogenesis in vitro through a gradient amplification mechanism. Proc Natl Acad Sci U S A. 2005 Nov 1; 102(44):15779-84.

2. Mac Gabhann F, Popel AS. Differential binding of VEGF isoforms to VEGF receptor 2 in the presence of neutropilin-1: a computational model. Am J Physiol Heart Circ Physiol. 2005 Jun;288(6):H2851-60.

3. Mac Gabhann F, Popel AS. Model of competitive binding of of vascular endothelial growth factor and placental growth factor to VEGF receptors on endothelial cells. Am J Physiol Heart Circ Physiol. 2003 Apr;286:153-164.