Pharmaceutical Research, Vol. 27, No. 4, April 2010 (© 2010)
DOI: 10.1007/s11095-009-0039-4

Research Paper

Characteristics of rhVEGF Release from Topical Hydrogel Formulations

Junyan A. Ji,[1,4] Jun Liu,[1] Steven J. Shire,[1] Tim J. Kamerzell,[1] Sung Hong,[1] Karen Billeci,[3] Ye Shen,[2] and Y. John Wang[1]

Received September 2, 2009; accepted December 14, 2009; published online February 13, 2010

Purpose. To study recombinant human vascular endothelial growth factor (rhVEGF), the release characteristics from topical gel formulations, and its interaction with the gelling agents.
Methods. The release kinetics were followed by quantifying rhVEGF that diffused into the receptor chamber of Franz cells. Analytical ultracentrifuge (AUC) was used to characterize the sedimentation velocity of rhVEGF experienced in the gel. The interactions were characterized by isothermal calorimetry (ITC), and rhVEGF conformation was assessed by circular dichroism (CD).
Results. The fraction of protein released was linear with the square root of time. The release rate constants did not show significant change within a wide range of bulk viscosities created by different concentrations of hydroxypropyl methylcellulose (HPMC) or MC gels. Sedimentation velocity determined by AUC generated comparable sedimentation coefficients of protein in these gels. AUC and ITC revealed no significant interaction between rhVEGF and HPMC and some change on secondary structure of the protein by Far UV CD, which was not the case with carboxymethyl cellulose (CMC).
Conclusions. Microviscosity, not bulk viscosity, was the key factor for the release of rhVEGF from cellulosic gels such as HPMC. Interaction between rhVEGF and CMC resulted in slower, and reduced amount of, release from the gel.

KEY WORDS: analytical ultracentrifugation; CD spectroscopy; CMC; diffusion; HPMC; hydrogels; isothermal calorimetry; methylcellulose; microviscosity; rhVEGF; viscosity.

INTRODUCTION

Over the past two decades, topical formulations of growth factors, such as basic and acidic fibroblast growth factors (FGF) (1–3), epidermal growth factor (4,5), rhVEGF (6), insulin-like growth factor (7), and transforming growth factor-β (8), have been explored for their potential applications in wound healing. Various material, such as alginate (6), gelatin (2), fibrin (7), polyethylene glycol diacrylate (9), polylactic and glycolic acid microspheres (10), polyvinyl alcohol (11), and collagen (12), have been included in these topical formulations. The water-soluble cellulose ethers, a family of gelling agents, have been the most commonly used excipients in hydrogel formulations because they are non-toxic, easily handled, sterilizable, inexpensive, and have a relatively simple manufacturing process. Both ionic and non-ionic hydrogels have been successfully used in different protein formulations (13). An anionic cellulose derivative, sodium CMC, is used as the gelling agent in a marketed platelet-derived growth factor (PDGF) gel (becaplermin [Regranex®]) (14), whereas non-ionic hydroxyethylcellulose (HEC) was used in basic FGF (1) and acidic FGF (3,15) gel formulations in clinical studies.

Recently, a topical formulation of rhVEGF was investigated for its potential therapeutic effect in wound healing of foot ulcers in diabetic patients (16). rhVEGF was expressed in *Escherichia coli* and purified as a covalent homodimer. Each monomer is composed of 165 amino acids, and the molecular weight of the protein is approximately 38 kD. Unlike most other topical products applied onto intact skin, the rhVEGF gel product was intended for direct application to an open wound. Tissue compatibility, safety, and sterilizability were, and continue to be, important considerations for product development. Cellulose derivatives, such as HPMC, MC, HEC and CMC, were considered the gelling agents of choice because these semi-synthetic cellulose derivatives have a long, successful history of use in ophthalmic preparations, skin protectants, vaginal gels, and open-wound products, as well as in oral sustained-release dosage forms.

For topically delivered therapeutics, the release of drug from vehicle is one of the essential parameters governing the bioavailability and efficacy of the product. In a topical

[1] Late Stage Pharmaceutical and Processing Development, 1 DNA Way, South San Francisco, California 94080, USA.
[2] Early Stage Pharmaceutical Development, 1 DNA Way, South San Francisco, California 94080, USA.
[3] Assay Technology Department, Genentech, 1 DNA Way, South San Francisco, California 94080, USA.
[4] To whom correspondence should be addressed. (e-mail: jji@gene.com)

ABBREVIATIONS: AUC, analytical ultracentrifugation; CD, circular dichroism; CMC, sodium carboxymethylcellulose; FGF, fibroblast growth factor; HPMC, hydroxypropylmethylcellulose; ITC, isothermal titration calorimetry; MC, methylcellulose; PDGF, platelet derived growth factor; rhVEGF, recombinant human vascular endothelial growth factor.

hydrogel formulation, both the gelling agent and the viscosity influence the diffusion and, subsequently, the release of drug. Drug diffusion rates in aqueous dispersions of polymers (gelling agents) are basically governed by the interaction and restrictive effects of the polymer on drug mobility. There is an inverse relationship between release or diffusion rates and gel viscosity, as predicted by the Stokes–Einstein equation (17,18). This trend was observed in the release of a series of benzoic acid derivatives from poloxamer 407 gels (19) and in the diffusion of methotrexate in Carbopol and poloxamer gels (20).

However, studies performed using dispersed hydrophilic cellulosic (21) and some non-cellulosic polymers (22) indicated that the drug diffusion rate scarcely changed over wide polymer concentration ranges with considerable variations in apparent viscosity. It has been suggested that this non-compliance with the Stokes–Einstein equation is because the effects of the polymer molecules on the macroscopic flow properties of the system do not necessarily correlate with the effects on diffusion in the same system. Al-Khamis *et al.* (23) suggested that the property known as microviscosity, measured by a dynamic light-scattering technique, should be used instead of macroviscosity or bulk viscosity as a predictor for the drug diffusion rate in systems of this type. This statement has subsequently been verified in several studies. De Smidt *et al.* (24) demonstrated that the macroviscosity of HPMC gel had a minor effect on the diffusion of theophylline. In another study (25), the diffusion coefficient of theophylline in a gel remained constant with hydroxypropyl cellulose (HPC) concentrations up to 0.8% but declined exponentially with the further increase of HPC concentration.

The diffusion behavior of small molecules in hydrogel is complicated, yet it may be even more complicated in the case of large molecule proteins in hydrogel. Although theoretical models of protein transport in hydrogels have been described and mechanisms proposed (26–28), there have been very few experimental data presented on the diffusion of protein in hydrogels. Fluorescence recovery after photobleaching (FRAP) was used to characterize the transport of large solutes, such as albumin (29) and FITC-dextrans (30), through biological tissues by measuring the diffusion coefficient. Liang *et al.* (31) studied bovine serum albumin diffusion in agarose hydrogel measured by the refractive index method and observed a slight decrease in release rate when the agarose gel concentration increased from 0.5% to 3%. The authors attributed this decrease, albeit very small, to the increase in gel viscosity.

Although numerous growth factors have been developed into topical gel formulations, little is known about the release of such proteins from the topical matrix. In the current study, the characteristics of protein release from hydrogel matrices were investigated, with attention to the manner in which viscosity may influence the diffusion rate. The release characteristics of rhVEGF from hydrogel formulations using HPMC at a wide range of viscosities with different protein concentrations were studied. The role of gelling agents was determined by using MC and HPMC to examine the effect of cellulose substitution and by using CMC to examine the effect of ionic charges on cellulose and rhVEGF release. In addition, the structure and interaction between protein and these cellulosic agents were investigated by CD, ITC, and AUC.

MATERIAL AND METHODS

Materials

Franz cells were purchased from PermeGear, Inc. (Bethlehem, PA). Polyvinylidene difluoride (PVDF) dialysis membrane, 1,000,000 Da molecular weight cut-off, was purchased from Spectrum Laboratories, Inc. (Rancho Dominguez, CA). Hamilton syringes, 250 μl, were purchased from Hamilton Company (Reno, NV). Positive-displacement pipettes and tips were purchased from Gilson, Inc. (Middleton, WI). HPMC (Methocel™ E4M, average MW: 86 kD, degree of methoxyl substitution: 1.90 and degree of hydroxypropyl substitution: 0.22) and MC (Methocel™ A4M, average MW: 86 kD, degree of methoxyl substitution: 1.75) were obtained from Dow Chemical Company (Midland, MI). Sodium CMC (CMC, medium viscosity grade, MW: 250 kD, degree of carboxyl substitution: 0.80–0.95) was purchased from Sigma-Aldrich (St. Louis, MO). A single lot of these cellulosic materials was used in this study. 0.9% Sodium Chloride Injection USP was purchased from B. Braun Medical, Inc. (Irvine, CA).

rhVEGF topical gels were made in-house from its liquid formulation at Genentech, Inc. (South San Francisco, CA). The liquid formulation was 5.0 mg/mL rhVEGF in a formulation buffer which contains 5 mM sodium succinate buffer at pH 5.0, 0.01% polysorbate 20, and 10.4% trehalose dihydrate. The gel formulation was made by mixing different ratios of the liquid formulation with 4.7% cellulosic material (HPMC, MC or CMC), which resulted in a final concentration of 0.2 mg/ml, 1 mg/mL or 1.8 mg/mL rhVEGF in 5 mM sodium succinate buffer at pH 5.0 and 3% cellulosic material with corresponding amounts of polysorbate 20 and trehalose dihydrate in various concentrations of cellulosic agents. All compositions in % are w/v.

In Vitro Release of rhVEGF from Gel Formulations

The release of rhVEGF from hydrogel formulations was measured using a Franz cell with a thermostat set at 31.5±1°C (to simulate skin temperature) under constant mixing. Six cells were run concurrently for each test gel. The Franz cell consisted of a jacketed cell with a 15-mm orifice diameter and a flat ground joint, clear glass with a 25 ml receptor filled with 0.9% sodium chloride solution, and a 14/20 Teflon stopper to prevent moisture loss from the donor cell. The clinical dosing regimen of rhVEGF hydrogel topical formulation for the treatment of diabetic foot ulcers was targeted at 120 μL per square centimeter of wound surface area for daily application. The surface area of a 15-mm (diameter) orifice in a Franz cell is 1.77 cm^2. Therefore, 212 μL of hydrogel, without other liquid, was loaded into the donor chamber in the Franz cell.

The hydrogel was transferred using a positive-displacement pipette set and evenly spread on a semi-permeable PVDF membrane. Samples from receptor chambers were taken every hour for 12 h, and the last sample was taken at 24 h. For each sample, 500 μL was taken from each receptor chamber through the sampling port and diluted 1:1 in ELISA assay buffer containing 0.5% bovine serum albumin. Afterward, the samples were frozen at −70°C until analysis by ELISA. The proper

reservoir level in the side arm was maintained by replenishing with saline solution so that there was no upward hydraulic pressure to bring normal saline from the receptor chamber to the donor chamber.

The release rate constants from the gels were determined from the slope of linear regression analysis of the relation between the fraction of rhVEGF released into the receptor chamber and the square root of time.

Bulk Viscosity Measurement

Viscosity of 3%, and other concentrations in the range of 1–4.5%, HPMC rhVEGF bulk gel was determined using a Paar Physica UDS 200 cone and plate rheometer (Graz, Austria) at a constant shear rate of 120 s^{-1} (about 20 rpm) using a 25 mm cone, temperature controlled to 25°C. A positive-displacement pipette was used to put approximately 200 μL of gel on the plate, and excess was removed once the cone was lowered into the measuring position. The average value of all readings at half-minute intervals over a span of 10 min was taken as the viscosity measurement. Typically, three independent measurements were taken for each sample. Each reported viscosity value was the average of three measurements for each sample.

Bulk viscosity of further diluted rhVEGF gels (i.e. 0.3%, 0.09% and 0.03% HPMC) and formulation buffer was determined using an Anton Paar Automated Micro Viscometer (Graz, Austria) with a 1.6 mm capillary and 1.5 mm falling ball at an angle of 60° with the temperature controlled at 20°C. Each reported viscosity value was the average of six measurements for each sample.

Density Measurement

Measurements of density were conducted using a precision density meter, DMA-5,000 (Anton Paar, Graz, Austria) with a precision of $\pm 1 \times 10^{-6}$ g/cm^3 and ±0.001°C. Solvent and sample solution densities were measured at 20°C. The density meter was calibrated with dry air and water for all temperatures prior to analysis.

Analytical Ultracentrifugation (AUC)

A Beckman Optima XL-I Analytical Ultracentrifuge (Palo Alto, CA) with UV and interference optical systems was used to study the sedimentation of the cellulose gels as well as the interaction between the rhVEGF protein and cellulose gels. The rhVEGF was mixed into HPMC gel yielding a final protein concentration of 1.0 mg/mL in HPMC concentrations of 0.3%, 0.09%, or 0.03%, respectively. The same study was also performed on a sample of 1.0 mg/mL rhVEGF in 0.3% CMC gel. A low concentration of HPMC or CMC was required because the high bulk viscosity prevented the introduction of solutions into the AUC centrifuge cell. Centrifuge cells equipped with double sector charcoal-filled epon centerpieces were filled with rhVEGF buffer and either rhVEGF- cellulose gels or cellulose gels without protein into the reference and sample sectors, respectively. Sedimentation velocity was performed at 40,000 rpm at 20°C or 25°C for analysis of rhVEGF mixed with cellulose gels, or 60,000 rpm at 25°C for analysis of cellulose gels, and concentration gradients measured using the absorbance scanning system at 280 nm for rhVEGF-cellulose solutions and interference optics for cellulose gel solutions. The sedimentation velocity data were analyzed using the Lamn equation fitting program Sedfit. Apparent sedimentation coefficients in solvent at temperature T, $s_{T,S}$, were corrected to sedimentation values in water at 20°C, $S_{20,w}$ using the relationship (32):

$$s_{20,w} = s_{T,S} \frac{(1-\overline{v}\rho)_{20,w}}{(1-\overline{v}\rho)_{T,S}} \frac{(\eta_{T,S})}{(\eta_{20,w})} \quad (1)$$

where $\overline{v}$ is the macromolecule partial specific volume in water at 20°C or solvent at temperature, T, $\rho_{20,w}$ is the density of water at 20°C and $\rho_{T,S}$ the density of the solvent at temperature T and $\eta_{T,S}$ and $\eta_{20,w}$ are the viscosities for solvent at temperature T and water at 20°C, respectively. The partial specific volume for rhVEGF, 0.71 ml/g, was computed using the additivity rule and amino acid composition. The partial specific volume for HPMC and CMC were not determined, and as a first order approximation a value of 0.72 cm^3/g for methyl cellulose in water was used (33).

The sedimentation coefficient of CMC-rhVEGF complex was calculated using the following equation:

$$s_{20,w} = \frac{(1-\overline{v}\rho)_{20,w} M}{f_{20,w}}$$

where M is the molecular weight of the CMC-rhVEGF complex, $f_{20,w}$ is the frictional coefficient which is calculated by assuming the CMC-rhVEGF complex has the same elongated structure as CMC monomer.

CD Spectroscopy

Far-UV CD was used to monitor the effect of different weight percentages of gelling agent on the secondary structure of the protein. CD spectra were recorded at 25°C using an AVIV spectropolarimeter (Lakewood, NJ) across a wavelength range of 250–190 nm with a 0.5-nm step, a 5-s averaging time, and a 0.1-mm path-length quartz cuvette. The concentration of rhVEGF was 1.0 mg/mL in the presence of 0.3%, 0.09%, or 0.03% w/v gelling agent. Protein secondary structure was estimated using the program CONTIN via the locally linearized implementation (34).

Isothermal Thermal Calorimetry

All calorimetric titrations were performed using a MicroCal VP-ITC Microcalorimeter (Northampton, MA) in both direct and inverse titration modes. rhVEGF and gelling agent solutions were prepared in formulation buffer. The calorimetric cell was filled with 1.6 mL of 1.8, 3, or 5.1 mg/mL rhVEGF, and the titration syringe contained 0.045% or 0.09% w/v gelling agent. A broad range of concentrations was used to encompass low and high molar ratios of protein to cellulose. The cell temperature was maintained at 30°C, and the solution stirring speed was 300 rpm. Heats of dilution were accounted for by subtracting the integrated heats of dilution from the binding heats. Integrated heats were fit to a

sequential binding sites model using three binding sites and a nonlinear least-squares Levenberg-Marquardt algorithm with Microcal Origin software.

RESULTS AND DISCUSSION

Flow Behavior and Release Characteristics of rhVEGF in HPMC Hydrogel

The rheological behavior of rhVEGF 3% HPMC gel is pseudoplastic. The viscosity decreased drastically with the increase in shear rate. The viscosity of the HPMC gel, with increasing concentrations of HPMC, was determined at a constant shear rate of 120 s^{-1} using a 25-cm cone. The viscosity of a 2.0%, 2.5%, 3.0%, and 4.0% HPMC gel was 1234 cP, 2336 cP, 3197 cP and 6389 cP, respectively. This trend shows that a slight change in HPMC concentration from targeted value of 3% could have resulted in a significant change in bulk viscosity of the gel. Additionally, the bulk viscosity is also affected by the polymeric properties of HPMC, such as degree of substitution. The viscosity specification for the 2% HPMC was set as wide as 3,000–5,600 cP at 20°C by the HPMC manufacturer. The lot-to-lot variation was reported by Dahl *et al.* (35), who showed broad variations in the viscosity of gels made from seven batches of HPMC provided by two different manufacturers. In addition, the HPMC gel viscosity also decreased with increasing temperature. The viscosity at 5°C, 30°C and 50°C was found to be 4094 cP, 2639 cP and 1997 cP, respectively. Because it is generally believed that the release rate is governed by the viscosity of the matrix (17–20), it is essential to evaluate how the variation in rhVEGF gel viscosity actually affects the release of the active ingredient, rhVEGF, from the gel matrix.

Fig. 1b shows the release profile of rhVEGF from 3.3% HPMC hydrogels at a protein concentration of 1.0 mg/mL. The release of rhVEGF from the gel through the semi-permeable membrane was most likely governed by the protein diffusion in hydrogel, since the pore size (1 million Da) of the membrane was much larger than the size of the rhVEGF molecules. In addition, absence of a lag time suggested that a rate-limiting step for protein release was not the permeation through the membrane, but rather its diffusion in the gel matrix. Protein diffusion through equili-

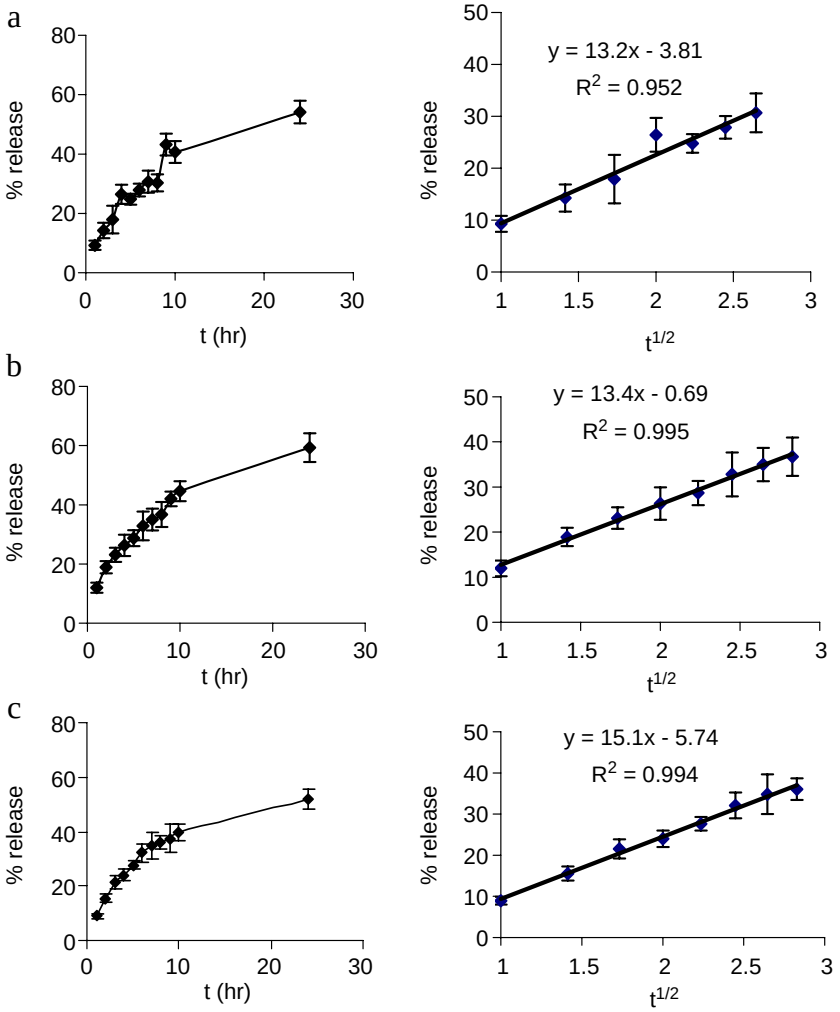

Fig. 1. Release profiles of 1.0 mg/mL rhVEGF HPMC gels and percent release relative to the square root of time ($t^{1/2}$). **a** 2.2% HPMC, **b** 3.3% HPMC, and **c** 4.3% HPMC.

brium gels can be described using the formula $M_t/M_\infty = k\ t^{1/2}$ to calculate the release rate constants, where M_t/M_∞ is the ratio of the absolute cumulative amounts of drug released at time t to the absolute amount of drug incorporated with the system at time $t=0$, respectively, k is a release rate constant incorporating structural and geometric variables and characteristics of the gel, and t is time (36,37). According to this equation, the cumulative percentage of drug released is in direct proportion to the square root of time. This type of square-root of time dependence was first reported by Higuchi (38) for suspended drug released from ointment. Al-Khamis *et al.* (23) reported the same pattern of release for salicylates from Carbopol gel. The plot of the percentage of drug released *versus* the square root of time of 1.0 mg/mL rhVEGF in 3.3% HPMC gel is shown in Fig. 1b. It shows that the cumulative fraction of rhVEGF released during the first 9 h exhibited a linear relationship with the square root of time (R^2>0.99). The slope is the release rate constant of rhVEGF from HPMC gel. The unit of the rate constant is percent per hour$^{1/2}$.

The release rate was further studied as a function of concentrations of gelling agent. Three concentrations of gelling agent in the formulation—2.2% (Fig. 1a), 3.3% (Fig. 1b), and 4.3% (Fig. 1c) HPMC—resulted in bulk viscosities of 960, 3242, and 6528 cP, respectively, at room temperature. Fig. 2 is the statistical analyses of release rate constants of 1.0 mg/mL rhVEGF in HPMC gels, and it showed no significant difference among all the samples with 95% confidence level. Table I summarizes the rhVEGF release rate constants at three HPMC concentrations and two protein concentrations (0.2 and 1.0 mg/mL) studied. Neither the gel viscosity within the range of 960 cP and 6528 cP nor the drug concentrations studied significantly affected the release rate. The differences among the determined values were within the range of variation of Franz cell experiments (39) and ELISA assays.

Comparison of rhVEGF Release Profiles Using HPMC, MC, or CMC as the Gelling Agent

Studies of alternative cellulose derivatives as gelling agents were conducted. Fig. 3 shows the release profile of rhVEGF using HPMC and MC as the gelling agents at a protein concentration of 1.8 mg/mL and using CMC as the gelling agent at a protein concentration of 1.0 mg/mL. In early clinical trials, 1.8 mg/mL rhVEGF in MC gel was tested. Based on the aforementioned result, release rate constants are independent of rhVEGF concentration, and therefore it is acceptable to compare the release characteristics of the three gelling agents at 1.8 and 1 mg/mL concentrations. There was no substantial difference between the drug release profiles from the HPMC and MC gel matrices, although the HPMC gel seemed to release slightly higher amounts of rhVEGF from its matrix (e.g., approximately 67% at 24 h, as compared with approximately 50% from the MC gel) perhaps due to the slightly higher hydrophobicity of MC than HPMC. It is noteworthy that the release from CMC gel was much slower than that from the HPMC and MC gels and that only 20% of the protein was released from the CMC gel at 24 h. CMC is an anionic polymer, whereas HPMC and MC are neutral polymers. The potential ionic interaction between gel and protein likely dominates in the case of the CMC gel. The viscosity of the 3% CMC gel was ~1200 cP, which was lower than the viscosity of two other gels (3% HPMC or 3% MC) studied. With this comparison, the slower release rate from CMC gel was not due to viscosity, since lower viscosity was expected to facilitate faster release rate. Thus, the slower release rate was attributed to ionic interaction between the CMC and protein. To evaluate the effect of protein and gel interaction on the rhVEGF release profile and rhVEGF molecular structure, AUC, ITC and CD studies were conducted. These experiments were done at lower gel concentrations because of the limitations of performing these biophysical experiments at higher gel concentrations. Nonetheless, investigation at the lower concentration of polymer can provide useful information regarding potential interaction of rhVEGF with the polymer molecules as will be demonstrated by the differences observed between HPMC and CMC in these studies.

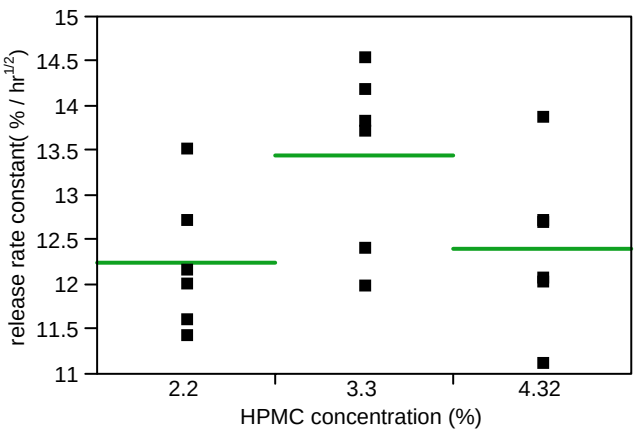

Fig. 2. Statistical analyses of release rate constants (% / hr$^{1/2}$) of 1.0 mg/mL rhVEGF in HPMC gels. The line is the mean value of the release rate constants.

Characterization of rhVEGF Interaction with HPMC Gel

The apparent sedimentation coefficient, $s_{T,\ S}$ is related to the corrected sedimentation coefficient in water at 20°C by the density and viscosity of the solvent (Eq. 1). The apparent sedimentation coefficient determined for rhVEGF at 1.0 mg/mL in formulation buffer at 20°C was 1.9 S. Correcting for the density and viscosity of the formulation buffer at 20°C (1.04 g/cc and 1.34 cP, respectively) using equation (1) yields an $s_{20,\ w}$ value of 2.8 S as shown in Table II. rhVEGF is a stable covalently bonded homodimer (40). Based on the crystal structure of a truncated form of rhVEGF (41), the theoretical $s_{20,w}$ of the dimer calculated using the program HYDROPRO (42) which generates a bead model, gives a value of 2.5 S which is consistent with the value of 2.8 S.

Sedimentation velocity analysis of the HPMC cellulose gel at 0.03%, 0.3% and 1.0% at 25°C shows that although the weight average molecular weight of the HPMC molecule is about 86 kD, the majority of the HPMC sediments with an apparent sedimentation coefficient less than 1 S (Fig. 4a). Correcting the apparent sedimentation coefficients using density and viscosity of the solvent, i.e., the formulation buffer at 25°C (1.03 g/cc and 1.18 cP, respectively), yields $s_{20,\ w}$

Table I. Release Rate Constants of rhVEGF from HPMC Gels at Different Viscosities and rhVEGF Concentrations

rhVEGF Concentration		HPMC Concentration 2.2%	3.3 %	4.3%
0.2 mg/mL	rhVEGF release rate constant (%/hr$^{1/2}$)	11.7	13.6	10.6
	Hydrogel viscosity (cP)	960	3242	6528
1.0 mg/mL	rhVEGF release rate constant (%/hr$^{1/2}$)	12.2	13.4	12.4
	Hydrogel viscosity (cP)	1212	3520	6372

values less than 1 S (Table II). This result is not surprising since sedimentation is directly dependent on molecular mass, but indirectly dependent on frictional coefficient as shown by the Svedberg equation (43). Flexible polymers such as HPMC would be expected to have a greater frictional coefficient than an equivalent 86 kD mass of a compact globular protein structure. Therefore, HPMC would have a lower sedimentation coefficient such as 1 S, whereas a globular 86 kD protein would have a typical value of 3 to 4 S. The sedimentation velocity analysis of rhVEGF in the presence of HPMC gels was done either at 20°C or 25°C. Fig. 4b shows the apparent sedimentation coefficient distribution at 25°C of rhVEGF in the presence of HPMC gel. The measured distribution at 20°C was similar and is not shown. The apparent sedimentation coefficients of rhVEGF at 20°C and 25°C are summarized in Table II. These apparent sedimentation coefficients are very close to the apparent sedimentation coefficient of rhVEGF at 1.9 S in formulation buffer, and suggest that there is no significant interaction between rhVEGF and hydrogels and the sedimentation of rhVEGF is occurring in the solvent which is the formulation buffer. This is consistent with the description often used of hydrogels consisting of a three-dimensional mesh-like structure where the spaces between the polymer chains are filled with water and small molecule solutes (44). The restriction of solute flow in these spaces is related to the pore size of the mesh relative to the size of the solute. Thus, the slight decreases in the apparent sedimentation coefficient as a function of HPMC concentration may be related to slightly greater restriction to flow in smaller pore sizes at the higher HPMC concentrations.

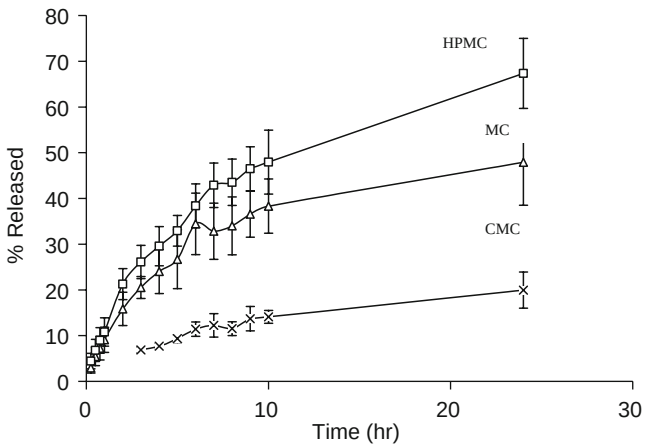

Fig. 3. Release profile of rhVEGF in 3% HPMC, MC, and sodium CMC. The rhVEGF concentrations were 1.8 mg/mL, 1.8 mg/mL, and 1.0 mg/mL, respectively.

Moreover, the rhVEGF appears to exist as a unimodal species; no noticeable amounts of larger or smaller species are observed. Correction of the apparent sedimentation coefficients to the standard condition of water at 20°C using Eq. (1) results in sedimentation values that are consistent with sedimentation of the rhVEGF dimer in formulation buffer. Altogether, these results suggest that there is no significant interaction between rhVEGF and HPMC gel at these concentrations and conditions. This phenomenon has been described in the literature (36,45). When the protein radius is much smaller than the pore radius of polymer, the diffusivity of protein in polymer is close to its diffusivity in water.

As described previously, the release rate constant of rhVEGF from HPMC hydrogel was not significantly affected over a wide range of bulk viscosities, as determined by a shear rheometer. The bulk viscosity measured by the viscometer generally is a reflection of resistance to molecular transport in solution. However, in a dispersion formulation of HPMC, the bulk viscosity does not necessarily represent the viscosity through which solute molecules travel, because the diffusion of solutes in polymers normally occurs through water-filled channels. Since rhVEGF was homogenously mixed with the HPMC in an equilibrium hydrogel, its diffusion was regarded as a slow, worm-like movement,

Table II. $s_{20,w}$ Values Corrected from the Determined Sedimentation Coefficients of 1.0 mg/mL rhVEGF with HPMC at 20°C and 25°C, and with CMC at 20°C in 5 mM Sodium Succinate Buffer at pH 5.0, 0.002% Polysorbate 20, and 2% Trehalose Dihydrate

	Study 1	Study 2	
	$s_{25,S}$ (S)	$s_{20,S}$ (S)	$s_{20,w}$ (S)
rhVEGF	ND	1.9	2.8
0.03% HPMC	0.8	ND	1.0
0.3% HPMC	0.6	ND	0.7
1% HPMC	0.3	ND	0.4
rhVEGF + 0.03% HPMC	2.2	1.9	2.9±0.1
rhVEGF + 0.09% HPMC	2.1	1.8	2.7±0
rhVEGF + 0.3% HPMC	1.9	1.6	2.5±0.1
0.3 % CMC	ND	0.4	0.5
rhVEGF + 0.3% CMC	ND	1.0	1.5
		1.4	2.1
		2.0	2.9
rhVEGF-CMC complex[a]	ND	ND	1.0 s

ND not determined

[a] The theoretical sedimentation coefficient of a complex of 1:1 molar ratio of rhVEGF dimer and CMC monomer, is calculated by assuming the complex contains the same elongated structure as CMC monomer

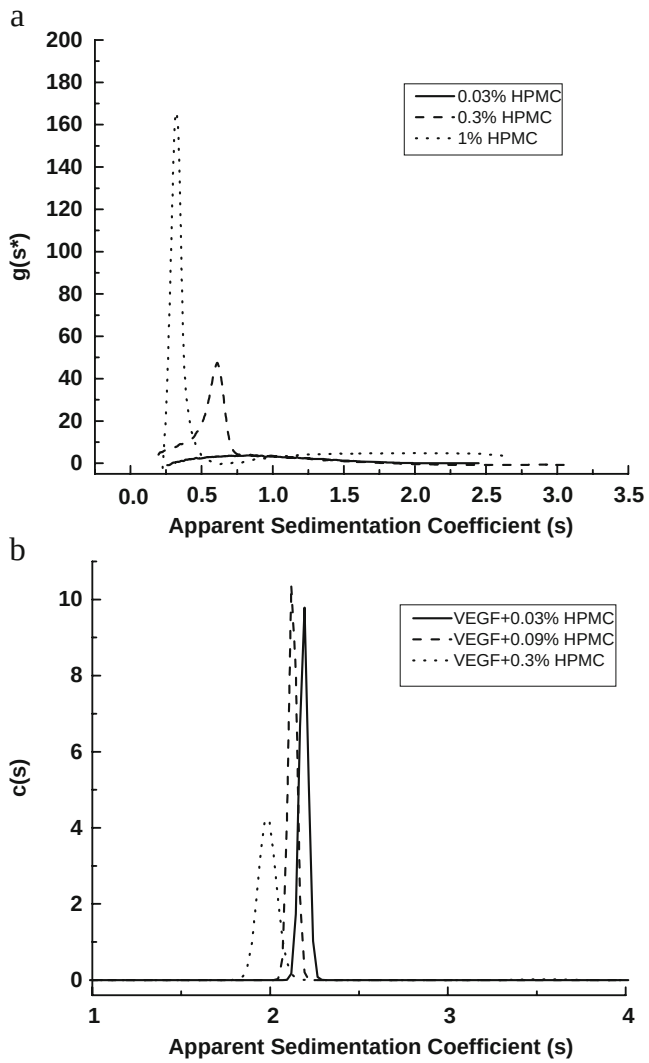

Fig. 4. a Sedimentation velocity analysis of HPMC at different concentrations at 25°C. solid line: 0.03% HPMC; dashed line: 0.3% HPMC; and dotted line: 1% HPMC. **b.** Sedimentation velocity analysis of 1.8 mg/mL rhVEGF with different amounts of HPMC at 25°C. solid line: rhVEGF in 0.03% HPMC; dashed line: rhVEGF in 0.3% HPMC; and dotted line: rhVEGF in 1% HPMC.

termed "reptation" through a polymer network (36). Therefore, it is microviscosity that represents the viscosity of the entrapped aqueous phase in the polymer network through which drug molecules diffuse. AUC was used for the primary purpose to assess macromolecular interactions, which directly impacts the mobility of rhVEGF through the cellulose gel matrix. AUC also resulted in a comparable conclusion regarding microviscosity as determined by studies of microspheres using photon correlation spectroscopy or light scattering (23,25).

The interaction between rhVEGF and HPMC was further studied by ITC, which typically provides a complete thermodynamic analysis of binding events. If the molecular interactions between rhVEGF and HPMC are moderate to strong, then the heat associated with the reaction would be directly observed. Little to no measurable changes in heat were observed when HPMC was titrated with rhVEGF over a broad concentration range (data not shown), suggesting very weak or negligible interactions. Binding enthalpy primarily reflects the strength of interaction between ligand and macromolecule relative to a blank solution and represents the enthalpy change of all non-covalent interactions including van der Waals, electrostatics and hydrogen bonding (46). Although changes in hydrophobic hydration can not be ruled out because the temperature dependence of enthalpy was not determined, a combination of data from AUC, ITC and the release kinetics suggests no large interaction between VEGF and HPMC.

Characterization of rhVEGF Interaction with CMC Gel

CMC is a negatively charged gelling agent, and HPMC is a neutral gelling agent, whereas the rhVEGF has a net positive charge at the pH 5.5 of formulation buffer. Thus, it was expected that interaction between rhVEGF and CMC or HPMC may play an important role in the difference of their release profiles. Fig. 5a shows the apparent sedimentation coefficient distribution at 20°C of 0.3% CMC. The distribution is quite broad, ranging from 2.5 S down with a peak maximum at about 0.4 S, likely reflecting on the heterogeneity of this polymer. Correction of the apparent sedimentation coefficient at the peak maxima yields a value less than 1 S, which again is expected, since, like HPMC, CMC is a flexible polymer with large frictional coefficient. The apparent sedimentation coefficient distribution for a mixture of 1.0 mg/mL rhVEGF and 0.3% CMC at 20°C is shown in Fig. 5b-c. The c(s) analysis in Fig. 5b shows two well-resolved peaks at 1.2 S and 1.4 S and a smaller leading peak at 2.0 S, while the g(s) analysis in Fig. 5c shows the similar trend, but with low resolution due to the diffusion effect. After correction using the solvent viscosity and density, the $S_{20,w}$ values for the two slower peaks are less than the corrected values for rhVEGF with and without HPMC. Since the detection during the centrifugation is with the absorbance system at 280 nm, only the sedimentation of rhVEGF or rhVEGF in complex with CMC is being measured, i.e., sedimentation of CMC itself is not monitored in this experiment. The two peaks at 1.4 and 2.0 S are likely due to rhVEGF that is associated to some degree with CMC resulting in a complex that essentially sediments more slowly than the more compact rhVEGF molecule because of the extended structure of the CMC polymer-rhVEGF complex. This result is further confirmed by a theoretical analysis where the calculated sedimentation coefficient of a hypothetical complex consisting of one CMC monomer and one rhVEGF dimer is significantly less than the sedimentation coefficient of a compact rhVEGF dimer (Table II). The smaller third leading peak has a corrected value of 2.9 S in very good agreement with rhVEGF sedimentation determined with and without HPMC. Thus, this peak represents the small fraction of rhVEGF that has not bound to CMC under these conditions.

A sequential binding site model was used to characterize the thermodynamic interaction of rhVEGF with CMC employing ITC (Fig. 6). The best fit sequential binding site model of the integrated heats allowed for 3 proteins bound per CMC unit with low to moderate affinities (Fig. 6). Favorable enthalpy components were measure for the first

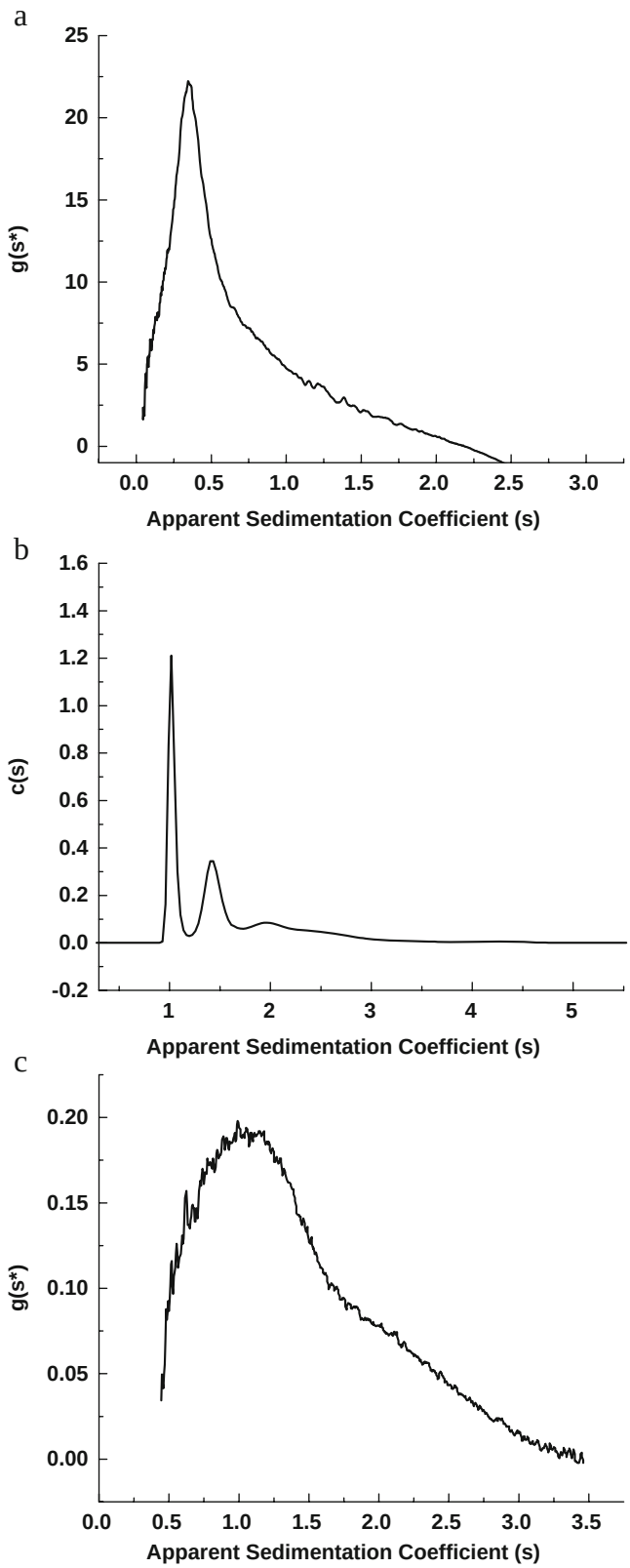

Fig. 5. **a**. Sedimentation velocity analysis of 0.3% sodium CMC at 20°C. **b.** Sedimentation velocity analysis of 1.0 mg/mL rhVEGF in the presence of 0.3% sodium CMC at 20°C. **c.** Sedimentation velocity analysis of 1.0 mg/mL rhVEGF in the presence of 0.3% sodium CMC at 20°C using dc/dt analysis.

(ΔH1 = −42 kcal/mol) and third site (ΔH3 = −140 kcal/mol), while the second site was endothermic (ΔH2 = 17 kcal/mol).

Numerous groups have studied the interaction of negatively charged polysaccharides, including heparin and dextran sulfate, with growth factors (47,54). Multiple proteins bind at high density to the charged oligosaccharide (~2–5 proteins to every 4–5 polysaccharide units) with moderate to high affinity. The exothermic enthalpy of interaction of rhVEGF with heparin has been reported as −33 kcal/mol (47), which is comparable to our observations for one site. In fact, much of the current literature describes the interaction of growth factors with sulfated polysaccharides, whereas our work describes the interaction of rhVEGF with CMC, which is presumably the result of positively charged protein patches interacting with the negatively charged carboxyl groups of CMC.

rhVEGF is a homodimer consisting of two receptor-binding domains and two heparin-binding domains. The structure consists of a short anti-parallel β-sheet and a short α-helix. Most of the surface positively charged amino acid side chains are localized on one side of the carboxy-terminal sub-domain, where the heparin domain is located, or on an adjacent, disordered loop near the amino terminus (41). CMC possesses negative charges because of the ionization of the carboxyl group at a formulation pH of 5.0 or above. The negative charge from the sodium CMC probably interacts with the heparin-binding domains, i.e., the regions of dense positive charge. In fact, our results are consistent with an interaction of two heparin domains (site1 and site3) binding to the carboxyl groups of CMC, and, hence, the favorable enthalpy of binding was observed for these two sites.

To characterize the effect of CMC on protein structure, CD spectroscopy was employed. The CD spectrum shows an intense band near 198 nm and a broad minimum between 200 and 210 nm, consistent with a structure consisting of β-sheets, strands and turns with some α-helical content (Fig. 7). Interestingly, the fit results of the CD spectra from the program CONTIN show residual loss of α-helix and increase in β-sheet and turn for rhVEGF in the presence of both CMC and HPMC (Table III). The percent change in secondary structure for rhVEGF is greater in the presence of CMC as

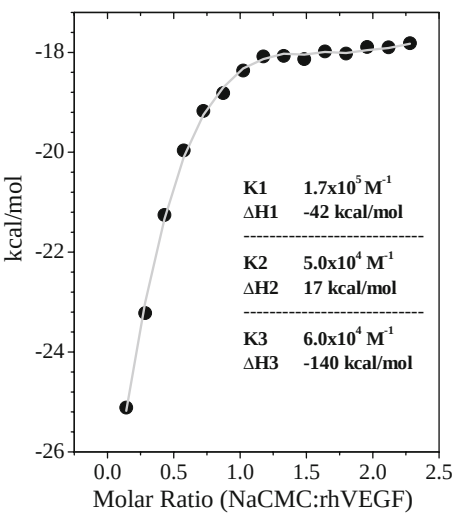

Fig. 6. Binding isotherm and best fit line using a sequential binding sites model of rhVEGF titrated with sodium CMC at 30°C.

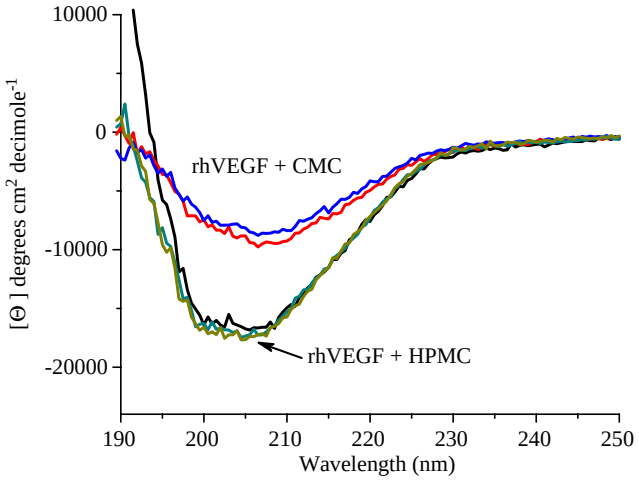

Fig. 7. Far UV CD spectra of 1.0 mg/mL rhVEGF alone (*black*), + 0.3% HPMC (*turquoise*), + 0.09% HPMC (*gold*), + 0.3% sodium CMC (*blue*), and + 0.09% sodium CMC (*red*).

compared to HPMC, as expected based on the results from AUC, ITC and the release kinetics. In fact, Brandner *et al*., also observed a modest decrease in the amount of α-helix and subsequent increase in β-sheet upon binding of glycosylated rhVEGF to heparin (47). The CD results for rhVEGF in the presence of HPMC suggest some change in protein structure which was unexpected based on the results from all other methods used to directly measure the interaction. However, it is unclear at this point how changes in CD signal relate to interaction with the polymers. In particular, the changes in signal may be related to subtle changes in environment in the hydrogel pores, or alteration of structure that occurs as the rhVEGF flows through the pores while intermittently contacting the polymer chain. At any rate, the degree of CD change does not necessarily reflect on the strength of the polymer rhVEGF interactions. Given that all the other methods used have not shown a strong interaction of HPMC with rhVEGF it is plausible to hypothesize distortion of rhVEGF structure as the molecule freely diffuses through the HPMC polymer network without direct strong interaction. Moreover, β-sheet structure has proven difficult to characterize using CD, which has been attributed to the dominating effect of α-helix signals and variations in the geometry of β-structure in polypeptides and proteins. In addition, the linear combination of secondary structures used in the approximation of secondary structure assumes equivalence of ensemble averaged structure and ignores geometric distortions of secondary and tertiary structure. The fitting procedures have been shown to provide much more reliable estimates of protein structure for α-helix rich proteins, while rhVEGF primarily consists of β-structure (48) suggesting potential inaccuracy in prediction. The large differences in the secondary structure prediction between rhVEGF with and without polymer likely arise from the spectral changes in the low wavelength region (e.g., 190 nm) where the positive band from the α-helix structure dominates. Finally, it is well-known that precise measurements in the far-UV, 190 nm and below, can be problematic from instrumental limitations and absorbance interference due to buffers and molecular oxygen (49).

CMC also has been used in an FDA-approved PDGF product (Regranex Gel®) (14,50) as a gelling agent. It has not been reported whether PDGF, which has a heparin-binding domain like rhVEGF, exhibits impeded release from its gel matrix. The interaction between protein and matrix is not necessarily undesirable. Such an interaction was exploited by Jennings *et al.* (51), who used Carbopol, an anionic polymer with a greater number of charges per unit weight, to prepare a controlled-release gel formulation for heparin-binding basic FGF, which exhibited improved efficacy in an animal model.

CONCLUSIONS

The release of rhVEGF from HPMC hydrogel exhibited a linear relation with the square root of time. The release of rhVEGF was dictated by the diffusion rate, which followed the predicted equation. The changes in gel viscosity using different concentration of HPMC did not substantially affect the release rate. This independence of the diffusion rate and apparent bulk viscosity was attributed to microviscosity-controlled diffusion with a diffusate that was small in comparison to the interstitial space of the network of cellulosic material such as HPMC.

It has been reported that the diffusion of small molecules in a gel matrix is governed by microviscosity, not by bulk viscosity, because these molecules are much smaller than the interstitial space of the polymer network (23). It was uncertain whether a large molecule such as rhVEGF would obey this rule. To our surprise, rhVEGF also was governed by the microviscosity of HPMC when the nominal molecular weight of the HPMC was cited as 86 kD by its supplier, Dow Chemical.

The microviscosity to which rhVEGF was subjected was further supported by the AUC studies. The comparable apparent sedimentation coefficients obtained from gels with three different concentrations of HPMC, were similar to those obtained in the solvent without HPMC. Correction to standard conditions using solvent viscosity and density provides a self-consistent set of data that further supports the idea that the protein sedimentation occurs in the solvent which has a viscosity similar to water.

Table III. Estimation of rhVEGF Secondary Structure from Circular Dichroism

Protein ± Gel	% α-Helix	% β-strand	% Turn	% Unordered	RMSD[a]
rhVEGF in Formulation buffer	39	4	4	53	0.54
rhVEGF + 0.3% CMC	23	17	27	32	0.31
rhVEGF + 0.3 % HPMC	31	12	26	31	0.43

[a] RMSD is the root mean square deviations between the calculated and experimental CD spectra. The program Contin was used for all secondary structure estimates via the locally linearized implementation (32)

It has been reported that the bulk viscosity of a gel product can vary significantly according to the source of the polymer (23,52) or can be modified by the contaminating cellulase-like enzyme (53). Certain gel formulations need to be autoclaved to reduce their bioburden because they will be applied to an open wound or because proteins are susceptible to protease released from contaminating microorganisms. A significant amount of free radicals can be generated during autoclaving, leading to a decrease in viscosity after autoclaving and during long-term storage (50,52,55). The results presented in this report suggest that as for rhVEGF, or other proteins of similar properties, when formulated with HPMC gels, may not require a tight viscosity specification to ensure a particular release rate of drug.

Through AUC and ITC analyses, rhVEGF was found to exhibit no significant interaction with HPMC. In contrast, rhVEGF and CMC showed a significant change in binding by ITC, sedimentation coefficient distribution by AUC and CD spectra. The interaction most likely occurred between the heparin-binding domain of rhVEGF and the carboxylate moieties on CMC. The retarded release rate of rhVEGF from CMC gel may be explained by such an interaction.

ACKNOWLEDGMENTS

The authors thank Dr. Sherry Martin-Moe for supporting this study, Ms. Linda Khym for editing the manuscript, Dr. Tom Patapoff for helpful discussions, and Dr. Ann Daugherty and Ms. Erika Ingham for collaborating on the development of the gel formulations.

REFERENCES

1. Wang YJ, Shahrokh Z, Vemuri S, Eberlein G, Beylin I, Busch M. Characterization, stability, and formulations of basic fibroblast growth factor. Chapter 2 in Pearlman R, Wang YJ, editors. Pharmaceutical Biotechnology, Vol. 9, Formulation, characterization, and stability of protein drugs: case histories. Plenum, New York, 1996
2. Haraguchi T, Okada K, Tabata Y, Maniwa Y, Hayashi Y, Okita Y. Controlled release of basic fibroblast growth factor from gelatin hydrogel sheet improves structural and physiological properties of vein graft in rat. Arterioscl Throm Vas. 2007;27:548–55.
3. Volkin DB, Middaugh CR. The characterization, stabilization, and formulation of acidic fibroblast growth factor. Chapter 3 in Pearlman R, Wang YJ, editors. Pharmaceutical Biotechnology, Vol. 9, Formulation, characterization, and stability of protein drugs: case histories. Plenum, New York, 1996
4. Cho Lee AR, Leem H, Lee J, Park KC. Reversal of silver sulfadiazine-impaired wound healing by epidermal growth factor. Biomaterials. 2005;26:4670–6.
5. Grzybowski J, Oldak E, Antos-Bielska M, Janiak MK, Pojda Z. New cytokine dressings. I. Kinetics of the *in vitro* rhG-CSF, rhGM-CSF, and rhEGF release from the dressings. Int J Pharm. 1999;184:173–8.
6. Gu F, Amsden B, Neufeld R. Sustained delivery of vascular endothelial growth factor with alginate beads. J Controlled Release. 2004;96:463–72.
7. Nauman JV, Campbell PG, Lanni F, Anderson JL. Diffusion of insulin-like growth factor-I and ribonuclease through fibrin gels. Biophys J. 2007;92:4444–50.
8. Puolakkainen P, Twardzik DR, Ranchalis JE, Pankey SC, Reed MJ, Gombotz WR. The enhancement in wound healing by transforming growth factor-β_1 (TGF-β_1) depends on the topical delivery system. J Surg Res. 1995;58:321–9.
9. Gobin AS, West JL. Effects of epidermal growth factor on fibroblast migration through biomimetic hydrogels. Biotechnol Prog. 2003;19:1781–5.
10. Holland TA, Tabata Y, Mikos AG. Dual growth factor delivery from degradable oligo(poly(ethylene glycol) fumarate) hydrogel scaffolds for cartilage tissue engineering. J Controlled Release. 2005;101:111–25.
11. Bourke SL, Al-Khalili M, Briggs T, Michniak BB, Kohn J, Poole-Warren LA. A photo-crosslinked poly (vinyl alcohol) hydrogel growth factor release vehicle for wound healing applications. AAPS PharmSci. 2003;5(4):E33. doi:10.1208/ps050433.
12. Lin CC, Metters AT. Hydrogels in controlled release formulations: network design and mathematical modeling. Adv Drug Deliv Rev. 2006;58:1379–408.
13. Peppas N, Khare AR. Preparation, structure and diffusional behavior of hydrogels in controlled release. Adv Drug Delivery Rev. 1993;11:1–35.
14. Regranex Gel® 0.01% (becaplermin) [package insert]. Raritan, NJ: Ortho-McNeil, Division of Ortho-McNeil-Janssen Pharmaceuticals, Inc. May 2008. Available at http://www.jnjgateway.com/public/USENG/REGRANEX_Full_Labeling_PI.pdf. Accessed August 20, 2008.
15. Matuszewska B, Keogan M, Fisher DM, Sopper KA, Hoe C-M, Huber AC. Acidic fibroblast growth factor: evaluation of topical formulations in a diabetic mouse wound healing model. Pharm Res. 1994;11:65–71.
16. Ji JA, Borisov O, Ingham E, Ling V, Wang YJ. Compatibility of a protein topical gel with wound dressings. J Pharm Sci. 2009;98:595–605.
17. Shah AC, Nelson KG. Mass transport in dissolution kinetics. II: convective diffusion to assess role of viscosity under conditions of gravitational flow. J Pharm Sci. 1987;76:910–3.
18. Cheong LWS, Heng PWS, Wong LF. Relationship between polymer viscosity and drug release from a matrix system. Pharm Res. 1992;9:1510–4.
19. Gilbert JC, Hadgraft J, Bye A, Brookes LG. Drug release from Pluronic F-127 gels. Int J Pharm. 1986;32:223–8.
20. Lu G, Jun HW. Diffusion studies of methotrexate in Carbopol and Poloxamer gels. Int J Pharm. 1998;160:1–9.
21. de Smidt JH, Crommelin DJA. Viscosity measurement in aqueous polymer solution by dynamic light scattering. Int J Pharm. 1991;77:261–4.
22. Suh H, Jun HW. Physicochemical and release studies of naproxen in poloxamer gels. Int J Pharm. 1996;129:13–20.
23. Al-Khamis K, Davis SS, Hadgraft J. Microviscosity and drug release from topical gel formulations. Pharm Res. 1986;3:214–7.
24. de Smidt JH, Offringa JCA, Crommelin DJA. Dissolution kinetics of theophylline in aqueous polymer solutions. Int J Pharm. 1991;77:255–9.
25. Alvarez-Lorenzo C, Gómez-Amoza JL, Martínez-Pacheco R, Souto C, Concheiro A. Microviscosity of hydroxypropylcellulose gels as a basis for prediction of drug diffusion rates. Int J Pharm. 1999;180:91–103.
26. Deen WM. Transport in porous polymers. AIChE J. 1987;9:1409–16.
27. Peppas NA, Lustig SR. Solute diffusion in hydrophilic network structures. In: Peppas NA, Peppas NA, editors. Hydrogels in medicine and pharmacy, Vol. 1. Boca Ration: CRC; 1987. p. 57–83.
28. Pitt C. The controlled parenteral delivery of polypeptides and proteins. Int J Pharm. 1990;59:173–96.
29. Sniekers YH, van Donkelaar CC. Determining diffusion coefficients in inhomogeneous tissues using fluorescence recovery after photobleaching. Biophys J. 2005;89:1302–7.
30. Braeckmans K, Peeters L, Sanders NN, De Smedt SC, Demeester J. Three-dimensional fluorescence recovery after photobleaching with the confocal scanning laser microscope. Biophys J. 2003;85:2240–52.
31. Liang S, Xu J, Weng L, Dai H, Zhang X, Zhang L. Protein diffusion in agarose hydrogel *in situ* measured by improved refractive index method. J Controlled Release. 2006;115:189–96.
32. Schachman HK. Ultracentrifugation in biochemistry. New York: Academic; 1959.
33. Hinz H-J. Thermodynamic data for biochemistry and biotechnology. Berlin: Springer-Verlag; 1986.
34. Provencher SW, Glöckner J. Estimation of globular protein secondary structure from circular dichroism. Biochemistry. 1981;20:33–7.
35. Dahl TC, Calderwood T, Bormeth A, Trimble L, Piepmeier E. Influence of physico-chemical properties of hydroxypropylme-

thylcellulose on naproxen release form sustained release matrix tablets. J Controlled Release. 1990;14:1–10.
36. Peppas NA. Controlling protein diffusion in hydrogels. Chapter II.1 in Lee VHL, Hashida M, Mizushima Y, editors. Trends and future perspectives in peptide and protein drug delivery, Vol. 4. Boca Raton: CRC; 1995.
37. Siepmann J, Peppas NA. Modeling of drug release from delivery systems based on hydroxypropyl methylcellulose (HPMC). Adv Drug Delivery Rev. 2001;48:139–57.
38. Higuchi T. Rate of release of medicaments from ointment bases containing drugs in suspension. J Pharm Sci. 1961;50:874–5.
39. Bosman I. Transdermal delivery of anticholinergic bronchodilators: methodological and clinical aspects. University of Groningen, PhD thesis, Chapter 5: 65–78 (1996)
40. Fairbrother WJ, Champe MA, Christinger HW, Keyt BA, Starovasnik MA. Solution structure of the heparin-binding domain of vascular endothelial growth factor. Structure. 1998;6:637–48.
41. Muller YA, Li B, Christinger HW, Wells JA, Cunningham BC, de Vos AM. Vascular endothelial growth factor: crystal structure and functional mapping of the kinase domain receptor binding site. Proc Natl Acad Sci USA. 1997;94:7192–7.
42. http://leonardo.fcu.um.es/macromol/programs/hydropro/hydropro.htm
43. Cantor CR, Schimmel PR. Biophysical chemistry Part II: techniques for the study of biological structure and function. San Francisco: Freeman and Co.; 1980.
44. Amsden B. Diffusion within hydrogels. Mechanisms and models. Macromolecules. 1998;31:8382–95.
45. Hennink WE, Talsma H, Borchert JCH, DeSmedt SC, Demeester J. Controlled release of proteins from dextran hydrogels. J Controlled Release. 1996;39(1):47–55.
46. Freire E, Schon A, Velazques-Campoy A. Isothermal titration calorimetry: general formalism using binding polynomials. Methods Enzymol. 2009;455:127–55.
47. Brandner B, Kurkela R, Vihko P, Kungl AJ. Investigating the effect of rhVEGF glycosylation on glycosaminoglycan binding and protein unfolding. Biochem Biophys Res Commun. 2006;340:836–9.
48. Sreerama N, Woody RW. Computation and analysis of protein circular dichroism spectra. Methods Enzymol. 2004;383:318–51.
49. Johnson CW. Protein secondary structure and circular dichroism: a practical guide. Proteins: Structure, Funct Integr Genomics. 1990;7:205–14.
50. Chu PI, Doyle D. Development and evaluation of a laboratory-scale apparatus to simulate the scale-up of a sterile semisolid and effects of manufacturing parameters on product viscosity. Pharm Dev Technol. 1999;4:553–9.
51. Jennings RN, Yang B, Protter A, Wang YJ. Hydrogel composition for the controlled release administration of growth factor. US Patent 6,331,309 (2001)
52. Ji JA, Ingham E, Wang YJ. Effect of EDTA and methionine on preventing viscosity loss of cellulose-based gel. AAPS PharmSciTech. 2008;10:678–82.
53. Shahrokh Z, Beylin I, Eberlein G, Busch M, Kang L, Wong A, *et al.* Cellulose cleaving activity from *E. coli* detected in topical formulations of recombinant proteins. BioPharm. 1995;8:32–8.
54. Mach H, Volkin DB, Burke CJ, Middaugh CR, Linhardt RJ, *et al.* Nature of the interaction of heparin with acidic fibroblast growth factor. Biochemistry. 1993;32:5480–9.
55. Basumallick L, Ji JA, Naber N, Wang YJ. The fate of free radicals in a cellulose based hydrogel: detection by electron paramagnetic resonance spectroscopy. J Pharm Sci. 2008;98:2464–71.