Development of Macroporous Poly(ethylene glycol) Hydrogel Arrays within Microfluidic Channels

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Received July 14, 2010; Revised Manuscript Received September 19, 2010

The mass transport of solutes through hydrogels is an important design consideration in materials used for tissue engineering, drug delivery, and protein arrays used to quantify protein concentration and activity. We investigated the use of poly(ethylene glycol) (PEG) as a porogen to enhance diffusion of macromolecules into the interior of polyacrylamide and PEG hydrogel posts photopatterned within microfluidic channels. The diffusion of GST-GFP and dextran-FITC into hydrogels was monitored and effective diffusion coefficients were determined by fitting to the Fickian diffusion equations. PEG-diacylate (M, 700) with porogen formed a macroporous structure and permitted significant penetration of 250 kDa dextran. Proteins copolymerized in these macroporous hydrogels retained activity and were more accessible to antibody binding than proteins copolymerized in nonporous gels. These results suggest that hydrogel macroporosity can be tuned to regulate macromolecular transport in applications such as tissue engineering and protein arrays.

Introduction

Hydrogels are polymeric networks that are able to swell and retain aqueous solutions. These materials have been used to immobilize proteins in biological applications including tissue engineering, drug delivery, and chemical and biological sensors.1,2 Both naturally derived materials (e.g., alginites, chitosan, and dextran) and synthetic materials (e.g., poly(ethylene glycol) (PEG), poly(acrylic acid), and polyacrylamide) have been used to form biocompatible hydrogels with a three-dimensional structure characterized as a mesh.3 The hydrated microenvironment within hydrogels allows them to maintain the activity and structure of immobilized proteins.4 Hydrogels used as scaffolding materials to regenerate or repair tissue can also immobilize growth factors and extracellular matrix proteins important in cell signaling and proliferation.5 Enzymes have also been immobilized in hydrogels to form “smart” materials for diagnostics. For example, hydrogels containing immobilized glucose oxidase can swell in the presence of glucose and thus can be useful in monitoring blood sugar in diabetics.6 Protein-immobilized hydrogels have also been extensively applied in diagnostic devices such as protein arrays. Arrays of three-dimensional hydrogel elements that immobilize protein substrates or antibodies to quantify enzyme activity or analyte concentration often have higher sensitivity than proteins directly immobilized on a surface.7

Hydrogels have been used in microfluidic sensors and lab-on-a-chip devices with applications including biotin/binding, cell culture, and biological engineering.8—10 The microliter sample volumes required allows for the promise of a diagnostic that minimizes required reagent volumes and facilitates multiplexed measurements. However, development of robust assays to detect and diagnose diseases, such as cancer, has often been complicated by a lack of sensitivity and reliability of these assays and the ability to perform them cost-effectively. In previous work, we have investigated the use of hydrogel arrays to quantify the activity of tyrosine kinases involved in cancer, including BCR-ABL (chronic myeloid leukemia) and EGFR (breast, lung, and skin cancers).11—13 This assay directly quantifies the ability of enzymes in cell lysates to phosphorylate hydrogel-immobilized protein and peptide substrates.

An important design consideration for hydrogels used in biological applications is the transport of solutes through the hydrogel network, and the mesh size of the hydrogel relative to solutes is a primary factor that influences diffusion rates.14,15 The rate of drug release from a hydrogel delivery device depends on drug diffusion coefficients through the polymer mesh, which can be controlled by adjusting the hydrogel properties.3 Furthermore, hydrogels used to immobilize proteins in arrays for biological sensors and reactions have been investigated, and often rely on small molecules or low molecular weight proteins that are able to diffuse through the tight polymer mesh.16—18

Large macromolecular solutes do not penetrate hydrogel networks easily and the strong dependence of detection signal on the mass transfer of analytes is one of the main limitations for the sensitivity of microspot protein arrays.19 Large proteins can be readily encapsulated within a hydrogel network which permits rapid diffusion of small molecules. However, solutes with a hydrodynamic diameter comparable to the mesh size of a hydrogel will not be able to diffuse into the network.20 PEG-diacylate (PEGDA) hydrogels (M, 575—20000) have mesh sizes less than 0.1—10 nm.21—24 Similarly, the mesh size of neutral polyacrylamide gels with a monomer concentration greater than 4% (w/v) is less than 9 nm.25 The hydrodynamic radius (Rd) of monomeric BCR-ABL (185—230 kDa) and EGFR (180 kDa) are approximately 5—10 nm, based on a comparison to the known radii of several globular proteins of similar molecular mass.26 Increasing the mesh size or the porosity of hydrogels used in substrate immobilization arrays to quantify the activities of these kinases is expected to improve sensitivity.
Porosity can be introduced in a hydrogel to increase accessibility to large molecular weight proteins, and several methods have been reported, including the use of porogens, phase separation, and foaming. In this work, we investigated the use of poly(ethylene glycol) as a porogen to introduce porosity in low-monomer-density hydrogels photopatterned in a microfluidic channel and analyzed Fickian diffusion of proteins and large dextran molecules through the hydrogel. We found that the porogen induced macroporous morphology in hydrogels created from PEGDA. Furthermore, proteins immobilized in the interior of these macroporous PEG hydrogels retained their structure and were accessible for interaction with antibodies, in contrast with proteins immobilized in nonporous PEG hydrogels formed without porogens, which remained inaccessible to large proteins.

**Experimental Section**

**Materials.** Isobornyl acrylate, technical grade (IBA), tetraethylene glycol dimethacrylate (TEGDMA), 2,2-dimethoxy-2-phenyl-acetophenone, 99% (DMPA), poly(ethylene glycol), typical M_w 700 (PEG700DA), and 2-bromoethylamine hydrobromide, 99% (BEA) were purchased from Sigma-Aldrich (St. Louis, MO). Poly(ethylene glycol) diacrylate, polyacrylamide, and acrylamide, 99%, N,N'-methylenebisacrylamide, and glass microscope slides (Fisher) were supplied from Fisher Scientific (Waltham, MA). 1-[(2-Hydroxyethoxy)-phenyl]-2-hydroxy-2-methyl-1-propane-1-one (Ir-gacure-2959) was obtained from Ciba Specialty Chemicals (Basel, Switzerland) and prepared by dissolving 1 g in ethylene glycol (Fisher) to make 10 mL total solution. (3-Acryloxypropyl)-trimethoxysilane was purchased from Gelest (Morrisville, PA). Polycarbonate chambers, which have a 150 µm thick adhesive, were affixed to these glass slides and filled with a prepolymer solution containing an isobornyl acrylate (IBA) prepolymer solution, and illuminated with UV light through a transparency mask to photopattern microchannels. (b) A second mask is used to polymerize hydrogel posts within the microchannels. (c) Photograph of hydrogel posts within the microchannels (PEG700DA in the top two channels, polyacrylamide in the bottom channel). The dark spots in the top and middle channel and the spots in the bottom channel are shadows of the hydrogel posts. (d) Bright field image of a typical 4% polyacrylamide hydrogel spot. Scale bar represents 200 µm.

**Microchannel Fabrication.** Microchannels were fabricated using a similar method as one described by Beebe et al. Glass microscope slides were first cleaned in a 70:30 (v/v) mixture of H_2SO_4/30% H_2O_2 at 95 °C for 30 min. They were then rinsed thoroughly with Milli-Q water (Millipore) and dried at 140 °C. A silane coupling reagent was applied by immersing the slides in a 95% (v/v) ethanol/water solution containing 2% (v/v) (3-acryloxypropyl)trimethoxysilane for 2 min, then rinsing briefly with absolute ethanol and drying overnight at room conditions. Polycarbonate chambers, which have a 150 µm thick adhesive, were affixed to these glass slides and filled with a prepolymer solution containing IBA (1.9 mg), TEGDMA (0.1 mg), and DMPA (0.6 mg). The chamber was covered with a transparency mask (Figure 1a) and exposed to 320–500 nm light at 5.9 mW/cm² intensity for 10 s (EXFO OmniCure S1000). The unpolymerized material was removed by thorough flushing with ethanol. To ensure complete polymerization of the device, it was re-exposed to light as described above.

**Hydrogel Photopolymerization.** Hydrogel posts were photopatterned within microchannels in a similar fashion as described in the previous section (Figure 1b). The 4% (w/v) polyacrylamide prepolymer solutions were prepared as follows: 4 µL of 33% acrylamide mixture (3.12 g acrylamide and 85.8 mg bisacrylamide in a total volume of 10 mL) and 1 µL of 100 mg/mL of Irgacure-2959 (a water-soluble photoinitiator) in ethylene glycol were added to water for a total volume of 10 mL. The solution was allowed to flow into the channel by capillary flow or gentle syringe suction for viscous solutions. A transparency mask was placed over the chamber and held in place with a quartz slide. It was then exposed to 320–500 nm light for 600 s at 16.8 mW/cm² intensity. PEG8000DA hydrogel prepolymer solutions contain the same concentration of Irgacure-2959 as polyacrylamide prepolymer solutions and were exposed to 320–500 nm light for 60 s at 16.8 mW/cm² intensity. PEG700DA hydrogel prepolymer solutions were prepared with a total Irgacure-2959 concentration of 0.2 µg/µL and polymerized by exposure to 320–500 nm light for 60 s at 16.8 mW/cm² intensity. To increase porosity of hydrogels, PEG (average M_w 3400, 10000, 20000, or 35000) was dissolved in water to make a 30% (w/v) solution and was added to the prepolymer solutions at the desired concentration. After exposure to UV light, unpolymerized material was flushed from the chamber with deionized water.
were filled with glutathione transferase-green fluorescent protein (GST-GFP) or FITC-labeled dextran (average molecular weight of 3400 or 10000 by reaction with 2-bromoethylamine hydrobromide (BEA). Sodium hydroxide (6.2 N, 8 mL) caused phase separation when added to a solution of PEG (1 g dissolved in 4 mL of water). A solution of BEA (1 g) in water (0.5 mL) was then added to the basic PEG solution and vigorously stirred at room temperature for 24 h until a single uniform phase remained. The solution was then extracted with dichloromethane (3 × 10 mL), which was pooled and concentrated under air. PEG-diamine was precipitated with cold diethyl ether and dried at room temperature. The dried precipitate was dissolved and dialyzed in ultrapure water using 1000 MWCO cellulose ester tubing (Spectrum Laboratories) for 24 h to remove salts and unreacted PEG-diamine. PEG-diamine was then purified by extensive dialysis in ultrapure water with a 3500 MWCO dialysis cassette and freeze-dried as above.

Synthesis of PEG-Alexa Fluor 647 Conjugate. Amine terminal groups were introduced to poly(ethylene glycol) with average molecular weight of 3400 or 10000 by reaction with 2-bromoethylamine hydrobromide (BEA). Sodium hydroxide (6.2 N, 8 mL) caused phase separation when added to a solution of PEG (1 g dissolved in 4 mL of water). A solution of BEA (1 g) in water (0.5 mL) was then added to the basic PEG solution and vigorously stirred at room temperature for 24 h until a single uniform phase remained. The solution was then extracted with dichloromethane (3 × 10 mL), which was pooled and concentrated under air. PEG-diamine was precipitated with cold diethyl ether and dried at room temperature. PEG-Alexa Fluor 647 carboxylic acid, succinimidyl ester (Invitrogen) was conjugated to PEG-diamine according to the manufacturer’s instructions. Briefly, PEG-diamine (0.6 mg) was dissolved in 0.1 M NaHCO₃ (500 µL), to which a solution of Alexa Fluor 647 succinimidyl ester (0.4 mg) in DMSO (50 µL) was added and allowed to incubate overnight with stirring. The PEG-Alexa Fluor 647 conjugate was then purified by extensive dialysis in ultrapure water with a 3500 MWCO dialysis cassette and freeze-dried as above.

Immobilization of GST-GFP and Analysis of Function and Accessibility. Acrylic labeled GST-GFP fusion protein was included in the PEG prepolymer solution at the desired concentration, and hydrogel posts were photopatterned as described above. To assess protein activity, fluorescence was detected using an inverted epifluorescence microscope (Olympus IX70) and monochrome digital camera (Diagnostic Instruments SPOT RT). Protein accessibility was determined using standard antibody labeling techniques. A total of 5% (w/v) nonfat milk in TBST (10 mM Tris-HCl, 100 mM NaCl, 0.1% (v/v) Tween-20) was used to block nonspecific protein adsorption to channel surfaces. The primary antibody was rabbit anti-GST and the secondary antibody was Alexa Fluor 633 goat antirabbit from Invitrogen. Antibody fluorescence was detected using an inverted epifluorescence microscope and imaged using a field-emission scanning electron microscope (Hitachi S-900).

Characterization of Porosity. A functional analysis was used to determine the relative performance of different hydrogels in allowing macromolecular diffusion. Microchannels containing hydrogel posts were filled with glutathione S-transferase-green fluorescent protein fusion (GST-GFP) or FITC-labeled dextran (average M₅, 250000), and a drop of mineral oil was placed over the ports to minimize evaporation and convective flow. The diffusion into hydrogel posts was monitored with an inverted epifluorescence microscope (Nikon Eclipse TE300) and attached monochrome digital camera (QImaging EXI Aqua) by taking a time series of images. Hydrogel posts were polymerized as cylinders and appear as circular spots in microscope images (Figure 2a). Non-steady-state radial diffusion in a cylinder has previously been described by Crank. Assuming a constant surface concentration and that the diffusion coefficient is independent of concentration, the solution for the mean concentration in the cylinder at any time, t, during diffusion is

\[
\frac{C}{C_{\text{bulk}}} = 1 - \frac{4}{R^2} \sum_{n=1}^{\infty} \frac{1}{\alpha_n} e^{-\frac{D_{\text{eff}} \alpha_n^2 t}{R^2}}
\]

where \( R \) is the radius of the cylinder and \( \alpha_n \) is the nth-root of the equation \( J_0(\alpha_n R) = 0 \), which is a Bessel function of the first-kind and of zero-order. Macromolecule concentration was assumed to be directly proportional to fluorescence intensity, and eq 1 is equal to the ratio of the average fluorescent signal within the hydrogel spot to the average fluorescent signal in the area surrounding the spot. Therefore, the only variable in eq 1 is the effective diffusion coefficient, \( D_{\text{eff}} \), which was determined using a nonlinear least-squares fit to experimental data.

A second parameter that functionally describes porosity and the permeability of a macromolecule in the hydrogel is the relative spot intensity at equilibrium (Figure 2d). It was calculated by the ratio of the average intensity of the spot to the average intensity outside the spot when steady-state spot intensity was reached.

Preparation of Hydrogels for Scanning Electron Microscopy. Hydrogel slabs were prepared from prepolymer solutions of 5% PEG7000DA and 5% PEG7000DA/20% PEG35000. The prepolymer solutions were poured between two glass microscope slides with 4 mm glass spacers and exposed to 320–500 nm light for 60 s at 16.8 mW/cm² intensity. The hydrogels were then placed in water and allowed to swell overnight. Samples were dehydrated by passing them through a graded series of ethanol–water mixtures to 100% ethanol over two days and freeze-fractured in slush nitrogen. Fractured hydrogel samples were then dried by critical point drying (Tousimis Samdi 780), coated with platinum with a sputter coater (Sondraw Conductacat IV), and imaged using a field-emission scanning electron microscope (Hitachi S-900).
Macroporous PEG Hydrogels in Microfluidic Channels

Results and Discussion

Diffusion in Polyacrylamide Hydrogel Posts. Polyacrylamide prepolymer solutions were flowed into microchannels and hydrogel posts were photopatterned using a transparency mask (Figure 1a,b). Photopatterned polyacrylamide posts within microchannels are transparent (Figure 1c,d) and have previously been shown to have limited permeability to immunoglobulins and dextrans larger than 70 kDa. To functionally determine the extent of macromolecular penetration into the hydrogel spots, solutions of fluorescent solutes were flowed into the channels and spot fluorescence was monitored through time. Figure 2a shows typical images of GST-GFP diffusion in 4% polyacrylamide hydrogel spots immediately after introducing the GFP solution into the channels and after 40 min of diffusion. The relative spot intensities were calculated from similar images taken through time by calculating the ratio of the average fluorescent signal within the hydrogel spot to the average fluorescent signal in the area surrounding the spot. Figure 2b shows diffusion rates of GST-GFP fusion protein into polyacrylamide hydrogels with varying monomer concentrations and demonstrates that a mathematical model of simple diffusion fitted the experimental data well, with coefficients of determination ($R^2$) greater than 0.95. Low correlation would suggest that transport deviates from pure Fickian behavior as a result of gel swelling, interactions between the diffusing solute and the hydrogel, combined diffusion both through swollen networks and through water-filled pores, or convective flow. Typically, the model fits GST-GFP and 250 kDa dextran-FITC diffusion data in this study with $R^2$ of 0.950–0.996, which suggests that the Fickian diffusion model provides a good estimation of the effective diffusion coefficients in these hydrogels. The model deviates from experimental data when only trace amounts of macromolecular solute penetrate the hydrogel; an additional study would be needed to accurately describe diffusion in these cases.

The relative functional porosity of a hydrogel is a descriptive parameter of the mesh size, which is dependent on the density of a polymer network. A lower concentration of monomer or cross-linker typically forms a polymer with a larger mesh size and solutes diffuse through the network more rapidly. However, these low-density gels have lower mechanical strength. The minimum acrylamide concentration used in this study was 4% (w/v) because it formed a stable structure in our previous work with hydrogel arrays and lower concentration gels often detached from the glass support. The calculated effective diffusion coefficients for GST-GFP in 4–8% (w/v) polyacrylamide hydrogels are shown in Figure 2c. An increase in monomer concentration from 4 to 6% caused an expected decrease in the effective diffusion coefficient of GST-GFP ($p < 0.001$). An increase in acrylamide concentration beyond 6% did not result in a significant change in the diffusion coefficient.

The relative spot intensity (Figure 2d) is calculated as the ratio of the average fluorescent intensity of the spot to the average intensity of the surrounding GST-GFP solution (bulk). The value at steady-state is equivalent to the fraction of hydrogel volume that is accessible to a particular macromolecule and thus provides a measure of the relative porosity of the hydrogel. In 4% polyacrylamide hydrogel spots photopatterned within microchannels, less than 40% of the interior was accessible to GST-GFP, which has a molecular weight of 54 kDa. Therefore, these hydrogels have limited use for arrays which contain immobilized protein substrates used to quantify the activities of larger enzymes (e.g., BCR-ABL: 185–230 kDa) or interact with immunoglobulin G (IgG) antibodies (150 kDa). An increase in the accessibility to the interior of the hydrogel would allow a greater amount of immobilized substrate to be available for interaction with these large proteins.

Effect of Poly(ethylene glycol) Porogens in Polyacrylamide Hydrogels. To increase the accessibility of the interior of hydrogel spots to macromolecular solutes, we introduced porosity by using inert PEG as a porogen during polymerization. Methods to create pores within hydrogels during polymerization include the use of salts, organic solvents, and inert molecules. These porogens exclude the growing polymer chains from regions within the polymer volume and are removed from the hydrogel after polymerization. Another technique to form void space involves reacting carbonate salts with acids to form foam in the polymerizing mixture. However, proteins may denature or degrade in organic solvents, high salt concentrations, or acidic conditions. Therefore, many of these methods are not compatible with protein copolymerization. The use of PEG to increase porosity in 20% polyacrylamide has been investigated by Çaykara et al. The inert PEG occupies space within the polyacrylamide hydrogel and does not have a tendency to denature proteins. PEG has also been shown to stabilize proteins in microarrays. Its hydrophilic nature allows the PEG to dissolve in water and be removed from the hydrogel.

We examined the effect of PEG porogens on solute accessibility and diffusion rates in low density (4 wt %) polyacrylamide hydrogels. Figure 3a shows fluorescence images of 250 kDa dextran-FITC diffusion at steady-state in 4% polyacrylamide hydrogels containing 0–6% (w/v) PEG10000. Dextran of this size has a hydrodynamic radius ($R_H$) of 11.46 nm, which is larger than many proteins, including IgG ($R_H = 6.2$ nm) and fibrinogen ($M_T = 340000$, $R_H = 10.95$ nm). Although the diffusion of dextran is not comparable to the diffusion of proteins of similar molecular weight because of dissimilar hydrodynamic radii, they are frequently used as a macromolecular model to study permeability in tissues and membranes.

As the porogen content increased, we observed the anticipated increase in solute penetration (Figure 3c). Polyacrylamide hydrogels with 6% PEG10000 contained approximately 230% more 250 kDa dextran-FITC at steady-state than hydrogels without PEG porogen. However, the increase in porosity was coupled with a decrease in structural strength; hydrogels containing greater than 6% PEG10000 did not form or were easily dissociated from the channel. Spot size also decreased and macroporosity was observed in polyacrylamide formed with 6% PEG10000 (Figure 3a). Therefore, further improvement in the porosity of polyacrylamide hydrogels by using PEG porogens is limited.

Although the addition of PEG increases solute accessibility, it reduces the effective solute diffusion coefficient in the hydrogel (Figure 3b). To investigate this phenomenon, we fabricated hydrogels containing fluorescently labeled PEG porogens and quantified porogen transport from the hydrogel after polymerization. Fluorescence images of hydrogels prepared with PEG3400 or PEG10000 conjugated to Alexa Fluor 647 revealed that some porogen remained in polyacrylamide hydrogels after one day (Figure 4), and there was no significant change ($p < 0.05$) in the fluorescence intensity and hence PEG diffusion after 1 day (Figure 4e). Although a majority of PEG diffused from the hydrogel, some PEG remained as a semi-interpenetrating network (SIPN) with polyacrylamide. This SIPN
likely increases the local density of the hydrogel and hinders diffusion. Although solutes may quickly diffuse into the increased void space created by the porogen, diffusion through the bulk polymer may be hindered, resulting in reduced effective diffusion coefficients. The trade-off between enhanced substrate accessibility and reduced rate of diffusion caused by the addition of a PEG porogen to a polyacrylamide hydrogel is an important consideration that must be balanced by the expected enhancement of molecular interactions in the hydrogel caused by increased accessibility versus the mass transport limitations of solute–substrate interactions.

**Diffusion in Poly(ethylene glycol) Hydrogel Posts.** Poly(ethylene glycol) hydrogels have also been used to immobilize proteins.4,43 PEG has been widely used in biological systems as it is able to form a hydrogel which can maintain a hydrated environment around immobilized proteins.44 It has also been shown to resist protein adsorption, which is beneficial in reducing nonspecific background signal in protein arrays.45 Several studies have reported small-molecule diffusion in hydrogels made from concentrated solutions (>50%) of PEG-diacrylate (PEGDA).46–50 Diffusion of proteins has also been studied in PEG hydrogels with >10% polymer content.22,51–53 Although the transport of proteins larger than IgG has been investigated in PEG hydrogels, diffusion is limited.22 To our knowledge, there are no studies that have focused on significant diffusion of proteins larger than IgG and macromolecules through hydrogels prepared from PEGDAs. We investigated the effects of PEG porogens on PEGDA hydrogel structure to improve macromolecular diffusion in biological applications that require transport of large solutes through hydrogels.

Unlike polyacrylamide hydrogels, PEG8000DA hydrogels remained stable with porogen concentrations of up to 23% (w/v) PEG35000 porogen, which was the highest concentration and largest PEG we tested. Similar to polyacrylamide hydrogels, solute penetration in PEG hydrogels increased as the porogen content increased (Figure 5a). The effect of a smaller PEG3400 porogen on the porosity was not as profound when compared to PEG35000. This may be the result of the polymer relaxing and swelling to fill the void space left as the smaller PEG diffused from the structure. Concentrations as low as 4% PEG10000–AlexaFluor 647 conjugate were not detectable in the PEGDA polymer by fluorescence microscopy after washing the hydrogel posts for 2 days (Figure 5b). However, at higher concentrations, PEG10000 formed a SIPN, as seen in Figure 5c. The polymerization of diacylates forms heterogeneous gels that have areas of high cross-link density surrounded by areas of lower cross-link density.54,55 The larger PEG porogens may increase this heterogeneity by pooling in areas and exclude the polymerization of the diacrylate monomer. These unincorporated areas would be removed after washing the gels and results in a lower overall cross-linking density and higher porosity in the hydrogel.56

We attempted to polymerize dilute (1–10 wt %) solutions of PEGDA, with average $M_r$ ranging from 700 to 35000. Higher molecular weight PEGDA hydrogels have a greater mesh size and thus would appear to be more suitable for applications where macromolecular diffusion is desirable, such as antibody arrays and kinase assays.22 However, we have found that although PEGDAs with an average $M_r$ greater than 20000 could be successfully photopatterned within microchannels at concentrations as low as 4% (w/v), the resulting gels were not mechanically stable. When the unreacted prepolymer solution was washed from the channels, these hydrogel posts gradually swelled and dissolved. This suggests that the previously described phenomenon of a heterogeneous hydrogel with areas of unpolymerized monomer or unincorporated porogen has an effect on the structural integrity of the hydrogel. However, when the unincorporated polymer dissolves out of the hydrogel, the overall cross-linking and polymer density decreases, which weakens hydrogel structure.

**Macroporous Poly(ethylene glycol) Hydrogels.** Cross-link density is increased in polymers formed from lower molecular weight PEGDA compared with polymers of high molecular weight PEGDA. Hence, the mechanical stability and permeability of solutes in hydrogels is dependent on PEGDA size. In contrast with PEG8000DA, the morphology of hydrogels created with low molecular weight PEGDAs was altered upon the addition of porogens. Without porogen, 5% (w/v) PEG700DA
formed transparent hydrogels that appeared similar to hydrogels of both higher molecular weight PEGDA and polyacrylamide (Figure 6b). However, upon addition of PEG porogens at concentrations higher than 5%, the polymerized hydrogel became opaque and macroporous. The macroporosity of such a hydrogel can be observed under a light microscope (Figure 6a). SEM images of hydrogel slabs prepared from 5% PEG700DA with 20% PEG35000 porogen show a macroporous morphology (Figure 6c) in comparison with 5% PEG700DA formed without porogen, which shows a smooth nonporous structure (Figure 6d). The greater concentration of cross-links in PEG700DA hydrogels relative to larger molecular weight PEGDA hydrogels should result in a lower equilibrium water uptake. This may cause local PEG crystallization and phase separation between the polymerizing PEGDA and the bulk aqueous PEG mixture, a phenomenon known as “polymerization-induced phase separation” and has been studied in several hydrogels, including polyacrylamide and PEG.\(^{37-59}\)  PEGDA hydrogels formed by polymerization-induced phase separation have been shown to be macroporous and retain their ability to resist protein adsorption.\(^{59}\)

The ANOVA test indicated that there was no statistically significant difference between the PEG porogen size and the effective diffusion coefficient of GST-GFP or 250 kDa dextran-FITC (Figure 6g). However, PEG porogen size did affect the amount of solute that diffused into hydrogel (\(p < 0.001;\) PEG3400 vs PEG35000). PEG3400 porogen yielded less solute-accessible hydrogels than PEG porogens with average \(M_r\) greater than 10000 (Figure 6h). With the use of equal concentrations of PEG porogen (20 wt %), the void space created should be equal despite differences in PEG molecular weight. However, PEGDA likely entraps some porogen during phase separation, causing incomplete porogen leeching and lower void volume. Lower molecular weight porogens such as PEG3400 may be completely encapsulated by the growing PEG700DA network. On the other hand, phase-separated areas in hydrogels with larger molecular weight porogen may be more interconnected, facilitating easier removal after polymerization and resulting in higher void space available for diffusion.

Macroporous hydrogels prepared from PEG700DA showed significantly greater permeability to large macromolecules, such as 250 kDa dextran-FITC (>60% accessible, Figure 6h), compared with polyacrylamide hydrogels prepared using PEG porogens (<40% accessible, Figure 3c). Interestingly, PEG700DA hydrogels with porogens of average \(M_r\) greater than 10000 resulted in a fluorescent signal in the interior of the hydrogel that was greater than that in the bulk (Figure 6e,f,h). Images from confocal microscopy showed that the fluorescence intensity in the center of the spot was less than the signal in the area surrounding the spot, which suggests that solute concentration in the hydrogel was not higher than in the bulk solution. Instead, the pores created from polymerization-induced phase separation in the PEG700DA hydrogel scatter fluorescent light, likely reflecting it to the objective and amplifying the signal.\(^{59}\) Although this yields a higher-than-expected signal or greater detection sensitivity, we assumed that the amplified signal is
proportional to the amount of solute diffused and the transport analysis is unaffected with average $R^2$ values for the fitted model above 0.95. Macroporosity is further verified by comparing antibody penetration and labeling of immobilized proteins in PEGDA hydrogels with nonporous polyacrylamide hydrogels that do not permit the diffusion of large macromolecules.

**Activity and Accessibility of Immobilized Proteins.** The macroporous morphology created in PEG700DA hydrogels by PEG porogens allows large macromolecules to penetrate the structure. These hydrogels, photopatterned within microfluidic channels, can be used to immobilize proteins that interact with binding partners or reaction substrates in solutions that are flowed through the channels. Concentration-dependent fluorescence of copolymerized GST-GFP fusion protein indicated that a significant amount of protein immobilized retained structural stability (Figure 7a). Furthermore, antibodies were able to interact with immobilized protein as demonstrated by an anti-GST antibody signal that is linearly ($R^2 = 0.941$) dependent on GST-GFP concentration (Figure 7b).

Substrate immobilized in the interior of macroporous PEG700DA posts was accessible for protein interaction (Figure 7a), in contrast to nonporous PEG700DA hydrogels formed without porogen which completely excluded IgG from penetrating beyond the hydrogel surface (Figure 7c). The intensity profile of the anti-GST signal across a macroporous 5% PEG700DA/20% PEG3400 hydrogel spot is compared to that of a nonporous 5% PEG700DA hydrogel spot in Figure 7d. Although the GFP signal was generally uniform throughout the spot, the antibody signal was most intense at the periphery of the spot. One possible explanation for this phenomenon is that the immobilized proteins are most accessible at the periphery, and the binding of multiple primary and secondary antibodies sterically hinders diffusion of additional antibodies into the interior of the spot. Furthermore, diminished antibody signal in the interior of PEG700DA hydrogels, despite a uniform GFP signal, may suggest that a majority of the immobilized GFP is encapsulated and not available to be labeled by antibodies. That is, only the GFP that is displayed on interior pore surfaces would be labeled, which may explain the heterogeneity of the antibody signal within the PEG700DA spots (Figure 7a, bottom). Further study is needed to investigate possible steric hindrance of antibody binding and additional enhancements in protein immobilization techniques to increase the amount of accessible protein.

Finally, the anti-GST signal was normalized to GFP fluorescence in nonporous PEG700DA spots and was compared with the signal in macroporous PEG700DA spots formed with 20% PEG3400 and 20% PEG35000 (Figure 7e). The signal in macroporous hydrogel spots formed with PEG3400 porogen was significantly greater than the signal in nonporous spots ($p < 0.05$) due to an increase in accessibility of the interior of the hydrogel. However, the signal in macroporous hydrogels formed from the larger PEG35000 porogen was not greater than the signal in macroporous hydrogels formed from PEG3400, despite diffusion studies showing improved diffusion. This suggests that there are other factors that influence substrate accessibility in hydrogels other than porosity. For example, during polymerization-induced phase separation with larger porogens, proteins may partition more into the interior of the gel and not be accessible for interactions at pore surfaces. In addition, the porogen size may affect the amount of protein encapsulated in the hydrogel and affect the ratio of accessible to immobilized protein. Despite this, macroporous hydrogels show increased signal intensity in the interior of the hydrogel compared with nonporous hydrogels and hence show promise in enhancing the sensitivity of a protein array assay. Alternate protein immobilization strategies such as
immobilization after polymerizing the macroporous structure may yield greater substrate accessibility and is a topic for further study.

Conclusion

In this study, we developed macroporous hydrogels that are photopatterned in microfluidic-based devices in the presence of PEG porogen. These macroporous hydrogels permitted significant diffusion of large molecular weight solutes into the interior of the gel compared with nonporous hydrogels. The macroporosity in PEGDA hydrogels arises from polymerization-induced phase separation. Hydrogels with insufficient cross-linking density and mechanical stability degrade under weak flow. However, the phase separation during polymerization yields a stable hydrogel post by condensing polymer into a more rigid but porous structure. In contrast to other studies, the low monomer concentrations used here increased solute permeability, but also resulted in unstable gels at very low concentrations. Although PEG porogens form SIPNs with polyacrylamide and PEGDA hydrogels, a heterogeneous network is created and porosity is increased after a majority of the porogen leaves the structure. The results presented in this work show that inducing macroporosity in hydrogels leads to improved solute transport. The increase in transport to the interior of the gel would allow greater biomacromolecular interactions with immobilized targets and suggests the ability to tune material properties to regulate transport in biosensing and delivery applications.

Acknowledgment. We thank Megan Frisk for her expertise in microfluidics and John Yin for usage of an inverted epifluorescence microscope for the diffusion studies. We also thank Joe Heintz and the Biological and Biomaterials Preparation, Imaging, and Characterization Laboratory (University of Wisconsin–Madison) for SEM imaging. Funding for this work was provided by NIH/NIGMS Grant 1R01GM074691.

References and Notes
