Microfluidic Based Platform for Characterization of Protein Interactions in Hydrogel Nanoenvironments

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Hydrogel posts in microfluidic devices were investigated as reaction environments for characterizing protein interactions with the goal of mimicking the complexity of a biological environment. The hydrogel environment can be easily tuned to study specific properties of the biological environment. In this study, the hydrogel pore size was tuned to mimic the effect of confinement/crowding on protein interactions. Arrays of polyacrylamide posts of different cross-link ratios (4 and 10%) were fabricated inside microfluidic channels via photopolymerization. Fluorescence-labeled proteins (protein A (PA) and immunoglobulins (IgG)) were transported into the posts via diffusion, and their interaction was studied using FRET. As the pore size of the hydrogel decreased, the binding between the proteins was enhanced. The degree to which crowding enhances a binding interaction depends on the intrinsic properties of the proteins; we observed that, inside the hydrogel post, the PA–goat IgG affinity was increased more than PA–rabbit IgG affinity. The integration of controlled nanoenvironments (hydrogels) with controlled microenvironments (microchannels) provides enhanced parametric control for studying protein interactions, which would be beneficial in developing sensors, in diagnostics, and for mimicking the biological environment at both the cell and the tissue level.

The “omics” era promises a better understanding of biological processes at the molecular level, facilitating the development of more efficient diagnostic and treatment strategies. There is a growing need for tools that can characterize molecular-scale events with a greater degree of precision and in a high-throughput manner. Recent advances in microarray and microfluidics techniques have the potential to meet these requirements. In vitro characterization of protein interactions is typically performed in dilute solution (nM). On the contrary, the in vivo biological environment is complex as it encompasses a variety of macromolecules and their interactions; both specific and nonspecific. For example, the cytoplasm contains filamentous and globular proteins; the total concentration is 300-400 mg/mL. A common observation of the cytoplasmic environment is that the density of macromolecules is not consistent throughout the cytoplasm. Diffusion of non-interacting probes indicates that certain regions are densely packed compared to other regions. Moreover, the density of actin filaments (part of the cytoskeleton) is dynamically restructured during processes such as cell division and locomotion. The biophysical principles governing protein interactions in such a complex environment would be different from those acting in dilute solution. On the other extreme, studies performed inside cells can be difficult to characterize due to a multiplicity of interactions and heterogeneity between cells. Therefore, there is a need for model environments that are simpler than biological environments yet capture the basic characteristics of the biological nanoenvironment such as the presence of charge, crowding, and water content. Such a model would aid in the development of effective inhibitory molecules (e.g., drugs) in understanding the basic mechanisms of signaling.

As a first step toward creating such model environments, an array of 3D hydrogel structures (posts) placed in microfluidic channels is explored as reaction nanoenvironments. In the literature, crowded environments are mimicked using polymeric solutions (e.g., poly(ethylene glycol), PEG). The polymer chains occupy a certain volume and “exclude” the probe molecules from entering this space. This region is referred to as excluded volume. Experimental studies have shown that, in the presence of background molecules (e.g., globular proteins, polymers), there is an order of magnitude change in the affinity of proteins to DNA

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hydrgels of different compositions were fabricated via photopolymerization. The channels were 125 μm deep and 1000 μm wide. The hydrogels are cylinders of 125 μm in height and 300 μm in diameter.

**Figure 1.** (a) Enclosed cavity made using glass slide and coverslip separated by a double-sided adhesive tape. (b) Microfluidic channels and hydrogels inside. The channels were 125 μm deep and 1000 μm wide. The hydrogels are cylinders of 125 μm in height and 300 μm in diameter.

**EXPERIMENTAL SECTION**

**Device Fabrication.** The device was fabricated using liquid-phase photopolymerization. In this method, a cavity was first formed by adhering a glass coverslip (no.1) (Fisher Scientific) and a microscope glass slide (Fisher Scientific) using a double-sided adhesive (Grace Biolabs, Bend, OR) (Figure 1a). Access holes were drilled on the glass slide using a diamond drill bit. Through the access holes, the device cavity was filled with a prepolymer solution containing isobornyl acrylate (Surface Specialties), tetraethylene glycol dimethacrylate (cross-linker) (Sigma Aldrich), and dimethoxy-2-phenyl acetophenone (photoinitiator) (Sigma Aldrich). The device was irradiated with 365-nm wavelength at 18 mW/cm² intensity (Acticure; EFOS, Quebec, Canada) through a transparency mask, placed on the coverslip side of the device. The mixture was thus photopolymerized in designated areas to form the walls of the microfluidic channels (Figure 1b). The unpolymerized mixture was removed, and the channels were thoroughly flushed with deionized (DI) water and dried by placing on a hot plate at 35 °C for few hours.

**REFERENCES**

Table 1. Composition of Prepolymer Mixture Used for Fabricating the Hydrogels

<table>
<thead>
<tr>
<th>components</th>
<th>4% cross-linker “C4”</th>
<th>10% cross-linker “C10”</th>
</tr>
</thead>
<tbody>
<tr>
<td>acrylamide</td>
<td>0.48 g</td>
<td>0.45 g</td>
</tr>
<tr>
<td>bisacrylamide</td>
<td>0.02 g</td>
<td>0.05 g</td>
</tr>
<tr>
<td>4-(benzoylbenzyltrimethylammonium chloride)</td>
<td>5 mg</td>
<td>5 mg</td>
</tr>
<tr>
<td>N-methylldiethanolamine</td>
<td>5 mg</td>
<td>5 mg</td>
</tr>
<tr>
<td>total volume (DI water)</td>
<td>10 mL</td>
<td>10 mL</td>
</tr>
</tbody>
</table>

* The total monomer concentration in each composition was 5%. The two hydrogels differed in the ratio of cross-linker (bisacrylamide).

For the hydrogels, two different prepolymer compositions were prepared as summarized in Table 1. A series of hydrogel posts were fabricated in situ using liquid-phase photopolymerization as described in the previous paragraph. C10 hydrogel posts were first fabricated as they have better mechanical strength and can withstand shear stress caused by flowing solutions. After removing the unpolymerized mixture, the channel was flushed with DI water, rinsed, and filled with the prepolymer mixture for C4 hydrogel to fabricate a second set of hydrogel posts. Each channel contained six hydrogel posts, three of each type. The posts were thoroughly washed as described in the following section.

**Sample Preparation.** In order to thoroughly remove unpolymerized material from the hydrogels, the posts were soaked in DI water and heated at 90 °C for 10 min. Following this treatment, the posts were washed with phosphate-buffered saline (PBS) buffer solution. To minimize protein binding to polymer chains of the hydrogel posts and the channel walls, the channels were filled with 1% PEG 600 (Sigma Aldrich) and incubated for 1 h at room temperature. The posts were thoroughly washed with buffer solution and incubated in the buffer for 12 h (at room temperature) to ensure that residual PEG molecules are completely removed from the hydrogel posts. Protein solutions of various concentrations (200 nM, 1 μM) were prepared using Alexa 555-labeled rabbit (anti-goat) IgG (4 fluorophores/molecule), goat (anti-mouse) IgG (4 fluorophores/molecule), and Alexa 647-labeled protein A (5 fluorophores/molecule) (all purchased from Invitrogen and used as suggested by the manual) by diluting the stock solution in PBS buffer. The PEG-treated hydrogel posts were incubated in various mixtures of protein solutions for 15 h at room temperature before imaging.

**Fluorescence Imaging and FRET Analysis.** In this study, the FRET method described by Berney and Danuser was utilized. In this method, three sets of samples and seven micrographs are used to calculate the FRET signal. The three samples are and follows: (1) donor only, (2) acceptor only, or (3) both. Each of these samples was visualized using a confocal microscope (Bio-Rad Radiance 2100 MP Rainbow) with donor, acceptor, and FRET filter settings as described in Table 2. The micrographs are named such that the capital letter (D, A, F) indicates the filter setting and is followed by “d”, “a”, or “da” to indicate the presence of donor, acceptor, or both, respectively.

Table 2. Nomenclature Used in FRET Analysis

<table>
<thead>
<tr>
<th>sample</th>
<th>green HeNe laser (543 nm)</th>
<th>red diode laser (637 nm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>donor only (IgG)</td>
<td>Em: 590/70 Dd</td>
<td>Em: 650 Ad</td>
</tr>
<tr>
<td>acceptor only (PA)</td>
<td>Em: 590/70 Da</td>
<td>Em: 650 Aa</td>
</tr>
<tr>
<td>both (IgG and PA)</td>
<td>Em: 590/70 Dda</td>
<td>Em: 650 Fda</td>
</tr>
</tbody>
</table>

* Green HeNe laser and red diode were used as the excitation sources. HQ590/70 band-pass and 650 DCLXPR filters were used to collect the emission. D, donor; A, acceptor; F, FRET; Em, emission; PA, protein A.

The images were analyzed using Matlab. Each of the images was normalized, and the following calculations were performed (see Table 2 for nomenclature).

- donor coefficient (fraction of donor in FRET filter setting):
  \[ C_{\text{donor}} = \frac{F_d}{D_d} \]

- acceptor coefficient (fraction of acceptor in FRET filter setting):
  \[ C_{\text{acceptor}} = \frac{F_a}{D_a} \]

These coefficients were used to subtract the spectral bleed through from the raw FRET image (Fda) as

\[ \text{FRET} = F_d - C_{\text{donor}} \cdot (D_{da}) - C_{\text{acceptor}} \cdot (A_{da}) \]

and normalized FRET was obtained by

\[ NFRET = \text{FRET} / \sqrt{(D_{da} \times A_{da})} \]

**RESULTS**

A total of 18 experimental sets were carried out in triplicate with variation in the protein pairs (PA−glgG, PA−rlgG), protein concentrations, and nanoenvironments (solution phase and different pore sizes of hydrogels).

**Fabrication and Sample Preparation.** Hydrogels were fabricated from 5% acrylamide prepolymer solution containing 4 and 10% cross-linker ratios (Table 1). Both types of hydrogel posts were fabricated with an exposure time of 300 s. Due to higher degree of cross-linking, the C10 hydrogel post was smaller than the C4 hydrogel post (Figure 2c). Since confocal imaging was carried out after reaching equilibrium, this difference in size does not affect the FRET measurements. Protein concentration and FRET studies measurements for solution phase was performed in regions away from the posts within the same microfluidic channel. Low molecular weight poly(ethylene glycol) (PEG 600) solution was used to coat the channel walls and the polymer chains inside the hydrogel posts to prevent nonspecific binding.

**Distribution of Protein.** PEG-coated polyacrylamide hydrogel posts were incubated in protein solution (PBS buffer at room temperature) for 15 h. The fluorescence intensity inside C4 and C10 posts was 10–20% of the intensity in solution phase (Figure...
2a, b and columns labeled “D” in Figure 2c). However, the concentration of proteins within the hydrogel pores is rather high (10–100-fold) as estimated by assuming even distribution of pores in the post and accounting for volume occupied by the polymer chains (of the hydrogel). Between the hydrogels, the fluorescence intensity from C10 posts was lower than from the C4 posts. This difference in protein distribution could be due to the smaller pore size of the C10 hydrogels. PA being smaller in size (45 kDa) was present in higher amount than the immunoglobulins (150 kDa). In both C4 and C10 posts, there is a gradient in fluorescence intensity at the peripheral regions of the hydrogel. This gradient is most probably due to formation of lower cross-linking in the hydrogel arising from the edge effects during photopolymerization.

**Effect of $K_D$ on Protein–Protein Interaction.** The FRET intensity is indicative of the presence of the PA–IgG complex; higher intensity implies increased interaction between the protein pairs. In comparing the FRET signal in solution phase at equilibrium (open bars in Figure 3), we see a higher signal for the PA–rIgG pair for all protein concentrations. This trend corroborates with reported data; $K_D$ (equilibrium dissociation constant) for PA–rIgG is ~100 times lower than $K_D$ for the PA–gIgG pair.19

**Effect of Concentration on Protein–Protein Interaction.** Three different concentrations of the protein pairs were studied:

**Figure 2.** (a) Intensity profile along the horizontal diameter, showing the distribution of donor (0.2 μM) and acceptor (0.2 μM) proteins in and around a 4% hydrogel post. (b) Intensity profile along the horizontal diameter, showing the distribution of donor (0.2 μM) and acceptor (0.2 μM) proteins in and around a 10% hydrogel post. (c) Confocal images of hydrogel showing donor distribution and normalized FRET (NFRET) for PA and IgG proteins (5:1) in 4 (C4) and 10% (C10) cross-linked hydrogel. While the concentration of the donor molecules is lower inside the hydrogels than in the solution, the normalized FRET values are higher inside the hydrogels. Figure 3 depicts this difference in a quantitative manner.

**Figure 3.** Bar graph comparing normalized FRET in different experimental sets. The average intensity of 3 different hydrogels was taken. For the solution phase, the average intensity from 3 different regions (away from the hydrogel post) is reported.
DISCUSSION

In diffusion-controlled reactions in solution, such as the protein association reaction studied here, the reaction rate is proportional to the diffusion constant of the species. One would expect the reaction rate to decrease as the degree of crowding and confinement is increased because the diffusion constant decreases. However, our observation was to the contrary. A key feature in proteins is that the relative orientation of the reactants plays an important role. Although the proteins diffuse slower in the hydrogel, the confinement increases the chance of a reaction once the reactants are in proximity. This could be due to the increased sampling of favorable orientations of both reactants. In order to quantify the degree of confinement, we use two different types of hydrogel posts that have different average pore sizes. From data reported by Holmes and Stellwagen,\textsuperscript{23} the pore size in C4 hydrogel should be \(-60\) nm while in C10 hydrogel post, (the pore size should be \(-30\) nm).

The probability of a reaction can be estimated from a simple model. For simplicity, let us assume that the PA–IgG interaction follows a one-step reversible reaction as shown in eq 1. The frequency of collisions is inversely proportional to the cube of the container size in which the molecules are confined\textsuperscript{24} (Figure 4c–e). Therefore, the proteins in nanometer-sized pores of the hydrogel collide a billion times more often than in a micrometer-sized channel and \(-8\) times more often in C10 than in C4. Moreover, due to entropic contributions, the reduced space favors complex formation over dissociation.\textsuperscript{25} Thus, in a confined environment, the forward reaction rate \((k_f)\) is higher than the reverse reaction rate \((k_r)\) for a reversible reaction (eq 1). The contribution from crowding results in nonspecific interaction of the proteins with the polymer chains that could affect the activity of the proteins \(a_{PA}, a_{Ig}, a_{PA-Ig}\) in eq 2. Usually the activity coefficients \(\gamma_{PA}, \gamma_{Ig}, \gamma_{PA-Ig}\) are assumed to be 1 in dilute solutions, and \(K_D\) is obtained from the concentration of the proteins \((c_{PA}, c_{Ig}, c_{PA-Ig})\). In a crowded environment, the contribution from the activity coefficient cannot be ignored; thus, the actual \(K_D\) is a product of the activity coefficient of all species.\textsuperscript{17}

\[
\text{(1)} \quad \text{PA} + \text{IgG} \xrightleftharpoons{K_D} \frac{k_f}{k_r} \text{PA} - \text{IgG}
\]

\[
K_D = \frac{a_{PA}a_{Ig}}{a_{PA-Ig}} = \frac{c_{PA}c_{Ig}c_{PA-Ig}}{c_{PA-Ig}c_{PA}c_{Ig}} = \frac{1}{K_D^0} (2)
\]

In solution, PA has a higher affinity for rIgG than for gIgG by 2 orders of magnitude.\textsuperscript{15,26} In our results (Figure 3), we see that the PA has similar affinity for both proteins in C4 and has a higher affinity for gIgG in C10. Interestingly, a 300-fold increase in affinity between PA and gIgG was reported when the antibody was immobilized on a polyclayamide bead through an antigen;\textsuperscript{27} however, direct immobilization of gIgG did not increase the affinity toward PA. The increase in affinity of immobilized antibody–antigen complex was attributed to a decrease in diffusive dissociation rate and increased accessibility to the reactive surfaces. The basis for this argument can be found in a theoretical model

\text{REFERENCES}

developed by DeLisi\textsuperscript{28} that addressed the amplification of signals arising from ligand–receptor interaction and subsequent clustering on cell surfaces. A similar argument based on diffusion and accessibility can be used to explain the effect of confinement on reactive molecules within the pores of the hydrogel. We see greater enhancement for PA–glG than for PA–rIgG pairs probably due to better orientation of glG. Intrinsic properties of the antibody such as the bend architecture and the position of the hinge could affect the accessibility of the reactive site.\textsuperscript{29}

From our results, we can infer that the confining environments of hydrogel increase complex formation between interacting biomolecules. Certain interactions are favored over others; this is exemplified by the change in affinity of PA for glG over rIgG in C10. The degree to which the nanoenvironment affects the interaction depends on the pore size of the hydrogel, which in turn is controlled by the prepolymer composition. In this study, the two different types of hydrogels (C4 and C10) were fabricated sequentially. It is conceivable that arrays of hydrogel posts, differing not only in pore size but also in molecular composition and charge density, can be simultaneously fabricated in a microfluidic platform, by leveraging the benefits offered by microscale phenomena such as the slow mixing of laminar flow. By allowing the streams to mix by diffusion, a concentration gradient in monomer concentration can be generated along the length of the channel and subsequently create the posts by liquid-phase photopolymerization. This method will allow for fabrication of posts with different nanoenvironments in a single step. The microfluidic platform thus serves as a docking station for the hydrogel reaction chambers. Protein solutions can then be easily transported to the hydrogel posts using microchannels and various parameters such as concentration, pH, and the presence of small molecules can be tested in a high-throughput manner.

CONCLUSION

The hydrogel nanoenvironment allows for characterizing protein interactions in confined or crowded spaces. A possible future direction for using this platform would be to measure $K_D$ through a comprehensive analysis at various protein concentrations and to characterize the activity coefficient of the proteins inside the hydrogel post by using non-interacting proteins as the donor and acceptor molecules. Furthermore, studying nonreversible and enzymatic reactions would help to better characterize the hydrogel nanoenvironment. The integration of controlled nanoenvironments (hydrogels) with controlled microenvironments (microchannels) will allow us to carry out parameter sweeps to quantitively individual interactions within the cell (or tissue) more accurately. Photopolymerization\textsuperscript{21} allows for hydrogels of various compositions to be easily fabricated, thus giving a wide range in properties of nanoenvironments. The platform can be easily extended to studying other parameters (e.g., charge, presence of small molecule) that contribute to overall cell behavior. Thus, the curse of dimensionality presented by the spatiotemporal complexity of the biological environment could be dealt with by large-scale arrays of hydrogel structures. The ability to carry out such a comprehensive characterization of biomolecular interaction will complement systems biology approaches in understanding protein networks in cells. Some of the studies that can be performed on this platform include characterizing the influence of environment in protein folding and aggregation, and their role in conditions such as Parkinson's disease.\textsuperscript{30} The platform should allow for both hypothesis- and discovery-driven approaches in understanding biological processes at the molecular level.

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