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Towards Next-Generation Proteomic Assays: Functional Materials as Sieving Matrices and Binding Scaffolds

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ABSTRACT

Next-generation bioanalytical approaches for protein-level measurements are advanced by the integration capacity of microfluidic design strategies, as well as the fine fluid and material control possible. Photopatterning of polymers within fluidic volumes is a key tool in the suite of technologies available for seamless integration of assay measurement modalities, as well as rapid target detection. Here, we overview recent advances in heterogeneous and homogeneous immunoassays using functional polymers, electrophoretic transport, and microdevices.

INTRODUCTION

PA gel methods at the macro-scale are well established and extensively used in electrophoretic protein and DNA separation studies. The hydrophilic nature and tunable pore size of PA gels make this material indispensable for electrophoretic assays run in aqueous solutions. Here we detail two major classes of protein assays that are advanced through the combination of tunable polymer materials and electrophoretic analyte transport. Chiefly, we detail homogeneous electrophoretic immunoassays (mobility shift immunoassays) and heterogeneous immunoassays in which one binding partner is immobilized on a PA gel support.

Homogeneous electrophoretic immunoassays implemented in microfluidic formats are powerful techniques for the rapid quantitation of proteins in low volumes of complex biological fluids. In this technique, an antibody probe is used to specifically capture the analyte of interest and the free unbound antibody is electrophoretically separated from the bound immune complex allowing for analyte quantification. A successful electrophoretic immunoassay requires all analytes to be of the same net charge (either both positive or both negative). This limits the analytes that can be separated with this technique and most literature to date reports on a narrow range of weakly acidic proteins [1,2,3]. The development of a highly alkaline microfluidic immunoassay capable of separating analytes irrespective of their isoelectric (pI) point would eliminate the need to predict complex and antibody pI points and would expand the range of proteins detectable with this powerful technique.

One route to developing PA gels bearing custom functionalization is via incorporation of a streptavidin linkage on acrylamide monomers. When streptavidin-acrylamide (SA) conjugates are included with acrylamide monomer in a precursor solution, they become incorporated to the backbone of the PA chain during polymerization. This SA can then be linked to any biotinylated antibody. By controlling the relative concentration of SA, monomer acrylamide and biotinylated antibody within a gel precursor, one can specify the resulting gel and antigen binding site density across a porous matrix. PA gel porosity can be controlled with the adjustment of acrylamide

and bis-acrylamide concentration in the precursor solution [4]. Following the generic chip fabrication, streptavidin fixed microchannels can be custom immobilized with the desired biotinylated antigen or antibody.

THEORY

While cross-linked PA gels are established as protein sieving matrices, the material is also finding use as an immobilization support for capture moieties. In this section, we overview transport advantages afforded by 3D PA gels decorated with binding sites, implemented with electrophoretic transport of binding partner though the reactive gel.

Analyte Capture Efficiency is Boosted by 3D Reaction Site Matrix

We compare the capture efficiency of a functionalized polymer (PA gel) matrix to capture on the internal surface of an "open" capillary tube with 100 µm ID. The benefit of high immobilization surface area A_s is revealed by noting that the concentration of binding sites in a control volume *V* is $a_o = \frac{a_{o,s}A_s}{V}$ assuming a uniform site density $a_{o,s}$ (mol m^{-2}). Approximating the gel as a bundle of packed cylinders in simple cubic arrangement with radius equal to that of the mean pore radius of ~120 nm for a 4%T, 2.6%C PA gel [5] yields (via Equation 3):

$$\frac{\eta_{gel}}{\eta_{cap}} \sim \frac{A_{s,gel}}{A_{s,cap}} \sim 300 \qquad \text{Eq. 1}$$

Suggesting a ~2-3 order-of-magnitude increase in capture efficiency within the gel matrix as compared to the capillary surface. This estimate agrees well with our experimental observations of an ~180-fold improvement in η over that measured for capillary surface capture.

Microscale Mass Transport Accelerates Analyte Capture to Reaction "Speed Limit"

Probing of a target protein P_{target} immobilized within a 3D PA gel with antibody C electromigrating through the gel pores to form a stationary product immunocomplex can be framed as a homogeneous reaction occurring between two reactant bands mixed electrophoretically [5,6]. The arrangement of target protein on the polymer lattice circumvents the diffusion-limited mixing regime that often arises at the microscale. Here the appropriate mass transfer timescale is simply that of band/front crossing, $t_{cross} = \frac{w}{u_{rel}} \sim 2$ sec for probing of a captured target band, where *w* is its width and u_{rel} the velocity of the probe front.

In the case of target probing on the wall of a capillary, mass transfer and surface reaction rates can become intimately coupled via a surface boundary layer in which the probe is locally depleted [7]. The dimensionless factor that evaluates the interplay between surface reaction (rate coefficient k') and boundary layer mass transfer (rate coefficient β) is the Damkohler number:

$$Da = \frac{k'}{\beta} \qquad \text{Eq. 2}$$

Thus, for $Da \gg 1$, reaction outstrips mass transfer and the system becomes mass transfer limited, whereas for $Da \ll 1$, mass transfer outstrips reaction and the system is reaction limited. Estimating Da for the open capillary capture scenario reveals $Da \ll 1$ (at least for the low achievable values of p_{target}), reflecting reduced boundary layer resistances in confined micro-nanoscale reaction volumes.

The small values of *Da* and t_{cross} suggest that the minimum reaction timescale $\tau_R \sim \frac{1}{k_{on}c_o + k_{off}}$ controls the probing equilibration time, where c_o is the bulk probe concentration in the PA gel pores or capillary lumen and k_{on} and k_{off} are the forward and reverse binding rate constants, respectively [6]. As a rule of thumb, $k_{on} \sim 10^6$ M^{-1} s^{-1} and $k_{off} \sim 10^{-3}$ s^{-1} ($K_D \sim 1$ nM, depending on the antibody). Experimentally, we can choose the probe antibody concentration c_o to be in large excess compared to K_D at $c_o \sim 100$ nM, giving $\tau_R \sim \frac{1}{k_{on}c_o} = 10$ sec (*c.f.* $t_{cross} \sim 2$ sec). Further, at equilibrium, captured target species can be shown to be saturated with probe when $c_o \gg K_D$. The fact that the observed value of τ_R in several of our functionalize PA gel formulations is on the order of 5 min rather than 10 sec perhaps points to the fact that the kinetic "on" and "off" rates are modified in the gel environment [8]. Nevertheless, the rapid mass transfer regime operating in nanoporous PA gels accelerates the immunoprobing process to the reaction "speed limit".

RESULTS AND DISCUSSION

Heterogeneous Immunoassays in Electrophoretic Formats: Specific Antibody Detection

A canonical approach to heterogeneous immunoassays includes: 1) capture of the target protein on a solid substrate through an immobilized capture antibody, 2) detection with a matched pair detection antibody and introduction of secondary antibody, and 3) signal amplification via probes conjugated to the secondary antibody. While all three steps are crucial for a reliable and robust assay, the first step involving the efficient capture of target analyte in a complex fluid with minimal nonspecific capture of the other non-relevant biomarkers and proteins in the sample serves as the major bottleneck, which affects the success of the following assay steps. Recently immunoassays which utilize the high surface to volume ratio aspect of microfluidic techniques have been gaining increasing attention [9].

As shown in our past studies [1], PA gel matrices fabricated in microfluidic channels not only utilize the traditional benefits of microfluidic concepts but also further increase the target biomarker and capture analyte interaction, especially when used in conjunction with electrophoretic transport of targets to immobilized capture sites. Streptavidin acrylamide incorporated in a PA gel zone capture electromigrating biotinylated antibodies. Detection was on the order of seconds with exceptional capture efficiency (>90%).

In this work, we photopattern PA gels with and without SA in glass microchannel network. In regions of the device where SA is present, biotinylated target antigen is then electrophoretically delivered and immobilized as a means to subsequently detect antibody. As a demonstration, we use an immobilized antigen to detect antibodies to Hepatitis C Virus (HCV). Here, the antigen NS3 (in 1X Tris Glycine native buffer) and HCV antibody NS3 (spiked in 5% human serum diluted in 1X TG buffer) were used. As shown in Figure 1, first biotin conjugated HCV antigen NS3 was mixed with biotin and AF-488 conjugated NS3 (for tracking the immobilization) and electrophoretically injected and immobilized in the microfluidic chip in direction from 1 to 4. The immobilization step was monitored through the AF488 signal. In this case, the custom immobilization step was performed in 8 minutes including 5 minutes for loading of the antigen, 1 minute for the incubation and 2 minutes for washing-removal of the excess unbound antigens. Figure 1b shows the signal coming from immobilized NS3 antigens.

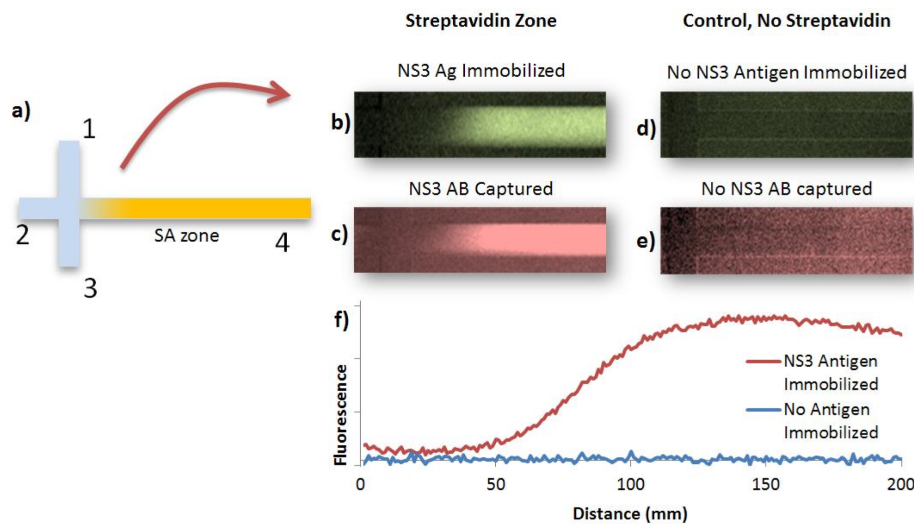


Figure 1. Heterogeneous detection of antibody using immoblized capture antigen in an electrophoretic heterogenous immunoasay. ***a)*** *Schematic of the microfluidic channel network. SA is only present in only in the region labeled "SA zone". Channels are 100 microns wide.* ***b)*** *Biotin-conjugated NS3 antigen is electrophoretically delivered from channel 1 to 4 and immobilized in the SA zone (signal from green spectrum filter).* ***c)*** *1ug/ml (6.6 nM) NS3 antibody with AF-568 labeling (spiked in 5% human serum) is electrophoretically delivered to the antigen immobilized zone from channel 2 to 4. A 10 minute loading is followed by a 2 minute wash (signal from red spectrum filter).* ***d)*** *No SA zone patterned, yielding no NS3 antigen immobilized.* ***e)*** *The negative control shows no antibody captured..* ***f)*** *Fluorescence signal along the channel for NS3 antigen immobilized (c) and no antigen immobilized (e) cases.*

To monitor detection of antibody by the immobilized antigen, AF568 labeled HCV NS3 antibody (6.7nM) was spiked in 5% human serum. NS3 antibody was then electrophoretically run against the antigen immobilized zone (from channel 2 to 4). Figure 1c shows the signal obtained from the captured target antibodies (under red spectral filter) after 10 minutes of sample load and 2 minutes washing. Signal distribution along the channel is shown in Figure 1f. The capture density reaches the highest levels closest to the boundary. Under these conditions, the capture signal depends on the sample load time, enabling detection even at clinically relevant low detection levels. Furthermore, secondary labeling strategies can be incorporated with the system such that system can be used in patient sera. Enzyme linked signal amplification methods can also be added to the assay if needed. Negative controls (Figure 1d and 1e) show the binding is specific. Comparison of the fluorescently labeled NS3 antibody concentration distribution for positive and negative controls is shown in Figure 1f.

Here we introduced a novel method for the custom immobilization of biotin conjugated antigens in a streptavidin fixed 3-dimensional PA gel matrix. The method enables custom modification of the generic fabricated microfluidic chips prior to the assay of interest. As the antibodies in the sample solution are captured in a three dimensional matrix, significant portion of the antibodies get captured close to the antigen immobilized and antigen absent boundary which leads to an enhanced signal potentially eliminating the need for a secondary label

associated signal amplification method. In this case, signal can be directly associated with the concentration of the target biomarker in the sample provided. Since the location of the immobilization boundary can be defined by channel network geometry or by the electric field, assay automation can be easily implemented.

Heterogeneous Immunoassays in Electrophoretic Formats: Integration with Separations

We sought to integrate immobilized capture biological materials (e.g., antigens, antibodies) with upstream electrophoretic protein assays.[10] To this end, we integrate the SA in gels designed and fabricated for polyacrylamide gel electrophoresis (PAGE). In this way, we realize fully contained protein immunoblotting, here native Western blotting. We have demonstrated integration of PAGE with subsequent heterogeneous immunoassays using a microchamber and channel network (Figure 2). Here, functional polymers allow us to define specific regions in the microchamber for sample loading, stacking, PAGE, and subsequent analyte capture.

Here, multiple PA gel regions were photopatterned within the central chamber (1mm x 1.5mm x 50 μm) of a glass microfluidic chip. Antibody functionalized precursor (containing photoinitiator) was wicked through the microchamber structure and the glass device was then aligned to a photomask on the stage of an inverted microscope. The masked chip was exposed to a UV light source directed through the objective lens (Fig. 2a). Unpolymerized solution was evacuated and the precursor for the next antibody gel region was introduced to the chamber by applying vacuum at adjacent reservoirs. The masking and polymerization process was then repeated until all requisite blotting regions were polymerized (Fig. 1b). Similarly, a 6% (w/v) PA separation gel and a lower density loading gel were patterned with an interface near the top of the central chamber (Fig. 1c, d).

As separated proteins electromigrate across the lateral span of the central chamber, target bands become immobilized as they cross the interface specific for their specific capture antibody partner. Figure 2 describes the migration velocity of three proteins as they move from left to right across the width of the chamber. The velocity of each protein band is calculated at multiple time points and plotted as a function of lateral position. The Protein G band comes to complete immobilization ($v = 0$ μm/s) within a distance of 0.6 mm, while C-reactive protein (CRP) becomes immobilized at 0.9 mm. These distances correspond to the blotting lanes containing antibodies directed against Protein G and CRP, respectively. Trypsin Inhibitor (TI) is included as a negative control and migrates across the chamber at constant electrophoretic velocity.

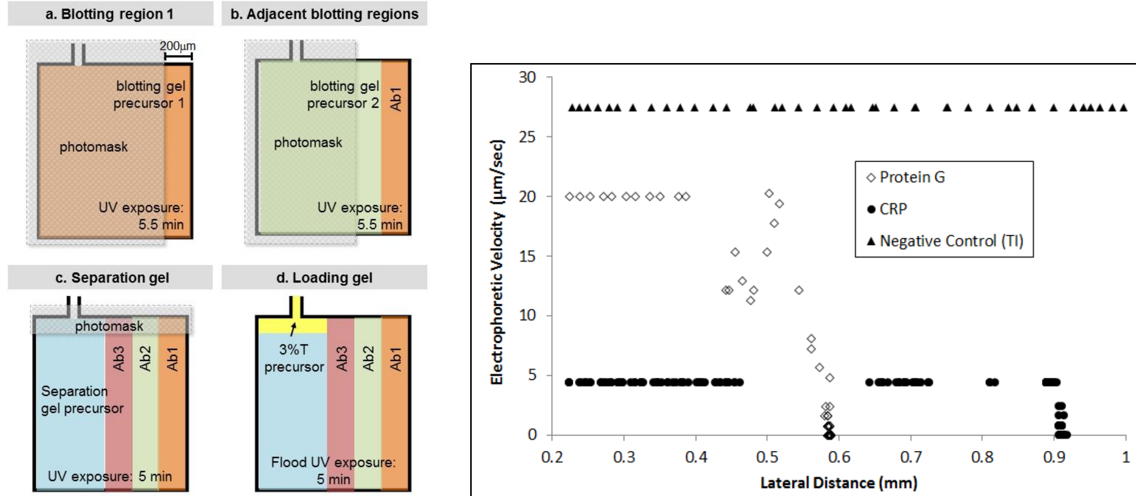
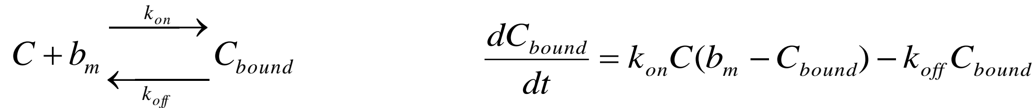


Figure 2. Photopatterned PA gels underpin integration of multistage electrophoretic assays in a microchamber, including here an embodiment of native Western blotting. (left panel) Multistep photolithography defines functional gel structures with high spatial resolution. (right panel) Analyte mobility is mapped to lateral location in the blotting regions and shows specific capture in regions where antibody partner is present. Negative control is not appreciably slowed.

The protein G band does not come to an abrupt stop at the boundary of the anti-Protein G gel (0.45 mm). Rather, the electrophoretic velocity gradually slows down until all antigen molecules are bound within 0.6 mm. This slowing of the band reflects a broad spatial distribution of the captured antigen, as the measured velocity reflects the aggregate migration for the band's center of mass. An examination of the Langmuir model of bimolecular binding kinetics (below) provides insight to the observed spatial distribution of the immobilized band.



$$C + b_m \underset{k_{off}}{\overset{k_{on}}{\rightleftharpoons}} C_{bound} \qquad \frac{dC_{bound}}{dt} = k_{on} C (b_m - C_{bound}) - k_{off} C_{bound}$$

Here, b_m represents the distribution of binding sites within the antibody functionalized gel. Analyte binding is governed by the association rate k_{on} ($M^{-1}s^{-1}$) and a disassociation rate constant k_{off} (s^{-1}). The non-dimensional Damköhler number (*Da*) represents the ratio of reactive flux (determined by k_{on} and binding site density) to the mass transport flux (electromigration). $Da=(Lk_{on}b_m)/U_o$ where L is the width of the blotting region and U_o represents analyte migration speed through the blotting gel. Specifically, $U_o=\mu_o E$, where E is the lateral electric field strength and μ_o represents the electrophoretic mobility of the analyte. *Da* describes the relationship between two timescales: electromigration time ($L/\mu_o E$) and binding reaction time ($1/k_{on}b_m$). At higher *Da*, the duration of protein target localization within the blotting gel exceeds the characteristic timescale for binding of most antigen molecules, so efficient binding results in shorter immobilized band distribution. At lower *Da*, the majority of target molecules move through the blotting region faster than binding occurs, and the reaction limited system results in a broader immobilized band distribution.

Homogeneous Immunoassays in Electrophoretic Formats

The aim of this work was to develop an alkaline homogeneous microfluidic immunoassay capable of making rapid, quantitative measurements of alkaline proteins using endogenous Lf in human tear fluid as a proof-of-principle marker. Lactoferrin (Lf) is a notoriously difficult-to-analyze, high abundance protein found in human tears, milk, saliva, serum, and mucosal secretions. Lf is a 77 kDa polycationic, highly alkaline protein with a pI point of 8.7.[4] In addition to its potential as a biomarker in ocular disease (e.g., Sjögren's syndrome),Lf has also been shown to have antimicrobial and anti-inflammatory activity in tears by reducing the availability of the iron necessary for microbial growth and inhibiting biofilm formation. Importantly, this work will expand the range of proteins able to be analyzed in a homogeneous electrophoretic immunoassay format.

Alkaline Buffer Selection and Effects

In order to impart a net negative charge on all protein analytes in the system, we employed a high pH buffer: N-cyclohexyl-3-aminopropanesulfonic acid (CAPS) titrated to a measured pH of 11. Operating at a pH of 11 maintains buffering capacity in the system while imparting a net negative charge on all analytes in the system with pI points < 11. Attention was paid to characterize the effect of the CAPS molecule and high pH conditions on the polymerization reaction and overall PA gel integrity. Full polymerization of all monomers in the precursor solution is critical as un-reacted acrylamide monomers can covalently bind to proteins at alkaline pH, decreasing the p*I* point of basic proteins and causing artifacts in basic PAGE.

Effect of pH 11 CAPS buffer on gel polymerization

PA gels are formed by copolymerization of acrylamide and bis-acrylamide (N,N-methylene-bisacrylamide) in a vinyl addition polymerization reaction initiated by a free radical-generating photoinitiator. Critically, Currie and co-workers [11] determined that, although the presence of alkaline buffers slows the propagation and termination steps, the overall polymerization reaction is not adversely affected by high pH conditions, given a constant initiation rate.

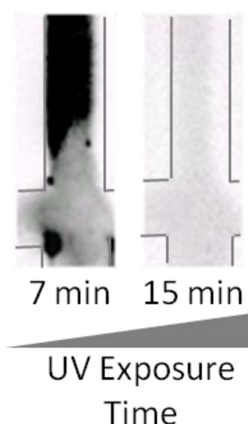


Figure 3. Increased polymerization time results in a reduction in protein-gel interactions

The introduction of the CAPS molecule has several effects on the polymerization rate equation. CAPS is a chain transfer agent that suppresses or retards polymerization by reacting with the initiating radical species and yielding radicals with low or no activity or capacity for further propagation. This serves to increase the rate of termination, decreasing the polymerization rate, shortening the overall chain length and reducing the mechanical integrity of the gel.[12] Experimentally, an increase in polymerization rate and gel mechanical integrity with increasing starting initiator concentration was observed. As a result of the predicted and observed reduction in polymerization rate, polymerization time was increased dramatically to 18 minutes (220% longer time than

polymerization in pH 8.4 Tris-glycine buffer) for all gels used in experimentation. It was found that the longer exposure times allowed for more monomer uptake and an overall reduction in analyte-gel interactions (Figure 3).

Alkaline hydrolysis of polymerized PA gels

The reduction in gel integrity caused by shorter chain lengths was further augmented by rapid alkaline hydrolysis of the polymerized PA gel which yields random copolymers of acrylamide and acrylic acid.[13] This build-up of negative charge decreases the analyte loading capacity and overall analyte mobility. As a result, only 25% of fabricated chips were usable and chip storage time was minimized to < 3 hours. BSA was also introduced as an internal velocity standard. A 30% reduction in starting velocity was set as a minimum threshold for acceptable performance.

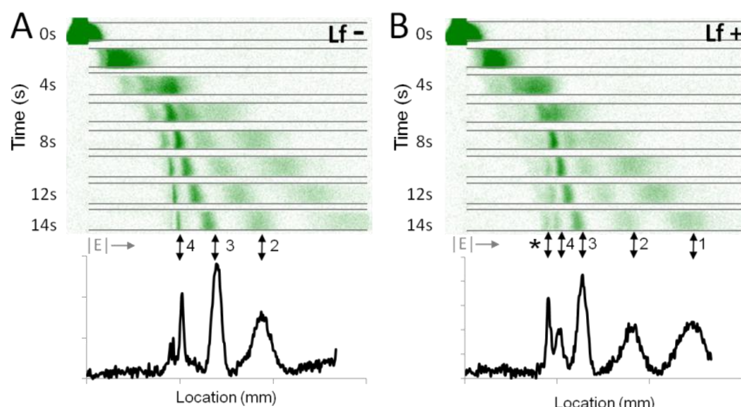


Figure 4. A highly alkaline buffer system allows for resolution of the lactoferrin immune complex and antibody in < 5uL of tear matrix. The assay completes in only 14 sec. Peak 1 = Free Dye, Peak 2 = trypsin inhibitor internal standard, Peak 3 = BSA internal standard, Peak 4 = anti-Lf antibody, complex is indicated with an asterisk.

Rapid Quantitation of Endogenous Lf

The final optimized assay incorporates a 3-6% pore-size discontinuity photolithographically fabricated *in situ* 0.36mm downstream of injection with pH 11 CAPS sample and gel buffers to resolve free antibody and immune complex (SR ≥ 1) in < 5 μL of dilute tear matrix and < 14 seconds of assay run time. Figure 2 compares separation montages of Ab* spiked in dilute tear matrix and Ab* + 283 nM Lf spiked in dilute tear matrix. An immune complex (indicated with an asterisk) appears when Lf is added. To our knowledge, this is the first demonstration of native PAGE at this high of a pH.

CONCLUSIONS

Microfluidic devices used in conjunction with directed electrophoretic transport show promise as a platform for conducting both heterogeneous and homogeneous immunoassays. In the former class of protein assays, PA gels underpin both high efficiency target capture and detection as well as integration of the heterogeneous immunoassay with upstream protein separations. In the homogeneous immunoassay, running buffer conditions have an impact on PA gel performance as a sieving matrix, demanding that the PA gel be optimized for high resolving power performance.

We see functionalized PA gels as a versatile and powerful material for next-generation protein measurements, as well as in realizing novel assays not possible on the macroscale.

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