

Detection of Antigens in Biologically Complex Fluids with Photografted Whole Antibodies

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A highly sensitive (pM), efficient ($t < 20$ min) detection assay was developed by designing surfaces with grafted antibodies. Through this approach, a short half-life antigen, glucagon, was rapidly detected in a biologically complex plasma/blood environment. Tailoring of graft composition eliminates the need for time-consuming blocking steps, significantly reducing antigen–antibody incubation times, while maintaining antibody specificity and activity toward target antigen. Grafted antibodies were bound through solvated, mobile polymer chains, thereby circumventing problems associated with antibody accessibility, analyte diffusion, and steric limitations. The efficiency of this assay is provided through grafting synthesized, acrylated antibodies in the presence of PEG monoacrylate. This procedure eliminates the need for blocking steps, due to a decrease in nonspecific protein interactions. These polymerizable antibodies were tethered with a range of densities while retaining biological activity. Moreover, biological activity of acrylated antibodies was compared to that of unmodified antibodies and remained comparable. The acrylated antibodies were grafted from substrate surfaces using controlled radical photopolymerization, maintaining the advantages of classical antibody immobilization techniques while providing improved detection. Through integrating this antibody conjugation chemistry and immunoassay approach with photolithographic techniques, construction of spatial patterns on a microfluidic device was demonstrated for efficient, parallel screening of multiple antigens.

Field schemes that facilitate the rapid detection (<20 min) of specific antigens greatly influence food and water processing, prompt toxin detection, and recognition of unstable, short half-life species.^{1–3} To date, a variety of laboratory-based immunoassay approaches have been developed for the detection of antigens,

including standardized enzyme-linked immunosorbant assays (ELISA), carbohydrate-based substrate assays,^{4–6} and a variety of other analytical, chromogenic, and fluorometric approaches.^{1–10}

Most antigen detection assays rely on monolayer formation or physisorption methods to immobilize antibodies to surfaces. However, many physisorption methods lead to drawbacks associated with antibody coating stability and uniformity and are often heavily dependent on substrate properties, such as surface chemistry and roughness.^{11–13} Thus, recent research has focused on methods to covalently bind antibodies to surfaces through conventional protein functional sites, such as amine, azide, and carboxy terminal groups, as well as antibody-specific thiol groups.^{11–19} For example, carbohydrate-based schemes have used thioether maleimide anchors to covalently attach saccharide groups to glass for the detection of complementary antibodies or antigens in a biologically complex environment such as plasma.^{4–6,20} While these approaches reduce the possibility of antibody desorption, surface-bound proteins generally lose much of their activity or selectivity due to conformational and mobility restrictions or mass-transfer limitations.^{11,13,16,18,21–23} The combination of

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many of these limitations has led to the inability to test for a variety of antigens that have decreased stability in a biological environment and also limits rapid detection capabilities.

In previous work, we introduced a method for synthesizing and photografting whole antibodies, focusing primarily on antibody acrylation and antibody grafting chemistries.³² In the aforementioned research and the work presented herein, acrylated whole antibodies were synthesized to establish a unique polymerization method to covalently immobilize antibodies from polymer surfaces in a manner that leads to increased accessibility and high mobility. Further, this approach enables covalent binding of the antibodies with independent control over their density and spacing, which improves detection sensitivity and response time. The acrylated whole antibodies were covalently tethered from surfaces via UV-initiated chain polymerization methods, specifically a living radical photopolymerization (LRP).^{24–27,32} LRP chemistry allows for spatial and temporal control over grafting and provides the ability to vary graft chemistry and composition to reduce nonspecific protein interactions or improve solvation of the grafted polymer chains. By increasing the mobility and accessibility of binding sites through surface grafted antibody chains, antibody–antigen binding is increased through extension of the functionalized chains into the analyte solution.

Herein, we highlight the use of photografted antibody architectures in the design of a rapid, highly sensitive detection platform that can further be introduced onto a microfluidic device for clinical diagnostic purposes. Also, rapid recognition with enhanced detection limits is achieved in phosphate buffer solution (PBS), plasma, and whole blood analyte environments by overcoming limitations of conventional immobilization techniques with respect to surface mobility and density. The decrease in assay time associated with using photografted antibodies is made possible by using PEG acrylate as the major component of the detection chain backbone. The PEG acrylate tethers prevent nonspecific protein interactions during antibody binding, so assay blocking steps that are normally necessary are eliminated. Ultimately, the decreased assay time makes possible the detection of biological molecules with short half-lives (<20 min),²⁸ as demonstrated herein by the detection of glucagon (GLGN) in a complex environment with sensitivities down to 1×10^{-12} M.

Integrating LRP photografting chemistry and antibody acrylation chemistry with photolithographic techniques allows for

construction of 3D microfluidic devices with integrated sensors.²⁹ Microfluidic detection devices constructed with this technique promote highly efficient, rapid, parallel screening of antigens. This contribution also demonstrates the promise that photografting has for facile introduction of multiple, dense ligand tethers with improved specificity and the ability to detect antigen otherwise undetectable with conventional techniques.

EXPERIMENTAL PROTOCOL

Materials. Antibodies including donkey anti-goat (DAG), rabbit anti-mouse (RAM), goat anti-rabbit (GAR), goat anti-rabbit HRP (GAR–HRP), and goat anti-mouse HRP (GAM–HRP) were purchased from ICN Biochemicals, Inc. (Irvine, CA). Monoclonal anti-GLGN was purchased from Sigma-Aldrich (St. Louis, MO). Glucagon antigen was purchased from Calbiochem (Jolla, CA). The antibody, peroxidase labeling kit was purchased from Roche (Indianapolis, IN). The 3,3'-5,5'-tetramethylbenzidine (TMB) staining kit was purchased from Corgenix Corp. The Vector VIP substrate was purchased from Vector Labs (Burlingame, CA). Trinitrobenzenesulfonic acid (TNBS) was purchased from Pierce (Rockford, IL).

Urethane diacrylate (UDA) Ebecryl 4827 was donated by UCB Chemicals Corp (Smyrna, GA). Triethylene glycol diacrylate monomer (TEGDA) was purchased from Sartomer (West Chester, PA). Poly(ethylene glycol (375)) monoacrylate (PEG(375) acrylate) and tetraethylthiuram disulfide (TED) were both purchased from Sigma-Aldrich. Poly(ethylene glycol) acrylate *N*-hydroxysuccinimide MW 3400 (ACRL-PEG-NHS) was purchased from Nektar Therapeutics (Birmingham, AL). The initiator, 2,2-dimethoxy-2-phenylacetophenone (DMPA) was purchased from Ciba Specialty Chemicals (Tarrytown, NY).

Synthesis and Characterization of Acrylated Whole Antibodies. Whole antibody proteins were acrylated by dissolving antibody at 6 mg/mL in 50 mM sodium bicarbonate, pH 8.4, and reacting the antibody amine groups with ACRL-PEG-NHS, MW 3400 in a variety of molar ratios ranging from 0.1 to 2.0 (NHS/NH₂). The reaction was allowed to proceed for 3 h at room temperature with shaking. Excess ACRL-PEG-NHS and other reaction byproducts were removed via dialysis against deionized water for 24 h (MWCO 10 000), followed by lyophilization, resulting in a solid product. All other polymerizable antibodies were synthesized using the same chemistry presented here.

Antibody acrylation was verified via SDS–PAGE and a TNBS assay.^{9,30} The degree of acrylation was determined using a standard TNBS assay protocol, monitoring the concentration of amine groups before and after conjugation chemistry was performed. Also, antibody digestion of disulfide bridges was performed to yield heavy (50 kDa) and light (25 kDa) chains. Then, SDS–PAGE was used to determine which regions of the antibody were being conjugated and further estimate the molecular weight of the fragments after being reacted with a range of stoichiometries of 0.1–2.0 NHS/NH₂ to perform a range of conjugations.

Determination of Functionalized Antibody Activity. Activity of modified antibodies was determined by using a standard ELISA protocol for the detection of a variety of antigen concentrations in PBS (5.0×10^{-11} – 5.0×10^{-8} M). RAM antigen was detected and compared to detection using both unmodified primary GAR and acrylated GAR antibody. The ELISAs were carried out on Immulon High-Binding 96-well plates. Each well was coated at 4

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°C overnight with 100 μ L of primary detection antibody (affinity purified, acrylated, and unmodified GAR) dissolved in 0.1 M sodium bicarbonate (pH 9.4) at a concentration of 5 μ g/mL. After aspirating off coating buffer and unattached primary antibody, wells were washed with 200 μ L of wash buffer (PBS + 0.1 v/v Tween-20). Each well was blocked for 2 h at room temperature, using 200 μ L of blocking buffer (PBS + 5 mg/mL fraction V BSA). After blocking, each well was washed 4 times with 200 μ L of wash buffer. Then, 100 μ L of RAM antigen in PBS (5.0×10^{-11} – 5.0×10^{-8} M) was added to each well and incubated for 1 h, followed by washing. After washing, 100 μ L of secondary GAR–HRP (5 μ g/mL) was added to each well for 1 h and incubated. Following appropriate washing, the wells were then tested for antibody activity using a plate reader (Victor², Perkin-Elmer) at 450 nm to detect activity between surface grafted antibody-containing chains and the complementary, enzymatic substrates. Activity was quantitatively analyzed through the use of TMB, a peroxidase substrate. Reaction of TMB with HRP resulted in a soluble blue product after 30 min, thereby creating a visually detectable chromogenic response in solution. At this point, the reaction between the TMB substrate and the HRP-labeled antibody graft was terminated through the addition of an equal volume of 0.36 N sulfuric acid, turning the TMB solution yellow for quantitative analysis by the spectrophotometer. All other standard ELISAs in this text were carried out using this procedure.

Substrate Preparation. Grafting substrates were prepared from monomer formulations consisting of 48.75 wt % aromatic UDA and 48.75 wt % TEGDA mixed with 1 wt % TED and 1.5 wt % DMPA initiator. The formulations were sonicated for 45 min and purged with argon gas for 2 min prior to photopolymerization. The substrate was then photopolymerized by exposure to a 45 mW/cm² intensity collimated, broad-range UV light (Hg arc lamp centered at 365 nm) for 500 s. After photopolymerization in the presence of TED, the resulting UDA/TEGDA, cross-linked polymer substrate has photolabile dithiocarbamate (DTC) groups that are further used to reinitiate the formation of surface-attached, photografted chains through LRP chemistry. These exposure conditions yield a polymeric network with over 90% double bond conversion, as observed when monitoring the acrylate double bond absorbance peak, using near-IR analysis.²⁹ Once polymerized, substrate samples are washed in copious amounts of methanol to remove any unreacted species prior to surface modification.

Photografting of Antibodies. Acrylated antibody was covalently photografted from a 2D polymeric substrate surface using the LRP surface chemistry. A solution containing 0.1 mg of acrylated antibody (includes mass of any protein impurities) was mixed with 1 mL of PEG(375) monoacrylate solution for 10 min and purged with argon for 2 min before grafting. A patterned region of grafted antibody/PEG(375) acrylate was formed upon exposure to 45 mW/cm² intensity UV light for 900 s using photolithographic techniques. The 900-s exposure time was used to optimize photografting, while still minimizing any protein damage due to UV exposure time as previously published.^{29,32} The resultant pattern was washed in 50/50 ethanol/water and then deionized water for 24 h, respectively.

Chromogenic Analysis of Photografted Antibodies. All antibodies were acrylated and purified via the coupling procedure. For example, acrylated GAR was patterned to form individual

detection squares (5 mm \times 5 mm—large size for visual contrast) on the substrate. After the specified reaction time, detection squares were rinsed 4 times with 100 μ L of PBS to remove any unbound antibody. After exposing grafted samples to RAM antigen at various concentrations in PBS (5.0×10^{-11} – 5.0×10^{-8} M) for 5 min at 37 °C, the samples were then washed and exposed to secondary GAR–HRP (5 μ g/mL) for 2 min at 37 °C. After washing 4 times with PBS, bound antigen was detected colorimetrically by GAR–HRP reaction with Vector VIP staining kit for 5 min. This reaction results in a surface-bound chromogenic response, where the amount of bound, HRP-labeled analyte is proportional to the intensity of the resulting purple color. After sample rinsing with PBS, quantitative gray scale analysis was performed using a digital scanner (Hewlett-Packard ScanJet 4100C) and NIH Scion Image analysis software. Control samples consisted of PEG acrylate grafted chains (with nonacrylated antibody washed away) assayed under the same conditions and a sample that was grafted using equal concentrations of antibody in the grafting solution but was not exposed to antigen during the assay procedure.

Detection of Unlabeled Antigen. GAR-grafted samples were prepared as described. After the appropriate washing was completed, a range of RAM dilutions (5.0×10^{-11} – 5.0×10^{-8} M) was prepared in PBS, and 100 μ L was placed on each antibody-modified sample square and incubated at 37 °C for 5 min. After four washes using PBS, 100 μ L of GAR–HRP at a concentration of 5 μ g/mL was added to each sample and allowed to react with the RAM for 2 min. After further washing with PBS, Vector VIP enzymatic chromogen (chosen for surface color contrast) was added and allowed to react for 5 min at 37 °C. Then, samples were rinsed and colorimetrically analyzed as described earlier using a scanner and NIH image analysis software. Control samples consisted of PEG acrylate grafted chains (with nonacrylated antibody washed out) assayed under the same conditions and a sample that was grafted using equal concentrations of antibody in the grafting solution but was not exposed to antigen during the assay procedure. These results were then compared to ELISA results that were determined using the standard ELISA protocol. (Figure 1).

Detection of Glucagon-Specific Antigen. Anti-GLGN grafted samples were prepared as outlined, with the exception of using a more concentrated grafting solution of 1.0 mg/mL anti-GLGN acrylated antibody in PEG. A range of GLGN dilutions was prepared in PBS, 20% whole blood in PBS, and 20% plasma in PBS. These solutions were tested on anti-GLGN grafted samples immediately after washing, due to the short half-life of GLGN (<15 min in whole blood)²⁸ in the presence of whole blood and plasma. GLGN–HRP was synthesized using a peroxidase labeling kit, purified using ultrafiltration, and then added to all GLGN dilutions at a concentration of 5 μ g/mL and allowed to react with the GLGN antigen for 2 min. After 5 min of reaction time at 37 °C, devices were rinsed and colorimetrically analyzed using Vector VIP chromogen exposure for 5 min. Gray scale analysis was then used to quantify results as described earlier. Control samples consisted of GLGN-grafted squares exposed to samples containing GLGN–HRP but no antigen and a PEG-acrylate only sample. GLGN ELISA data were gathered using the standard ELISA protocol.

Microfluidic Immunoassay Construction. Microassays with antibody-grafted detection wells were constructed using the

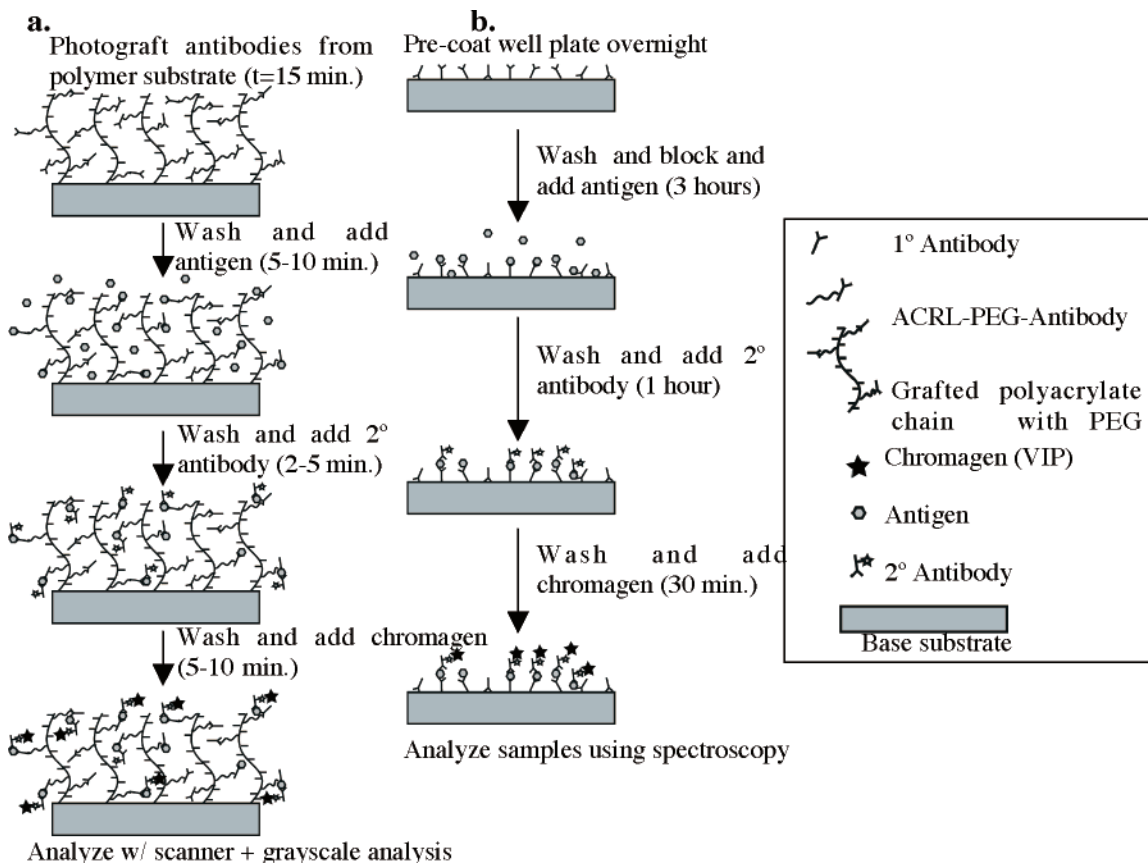


Figure 1. Detailed schematic of how covalently attached, photografted, antibody-containing tethers can be used to provide rapid detection of a specific antigen using a sandwich immunoassay approach (a) compared to a standard sandwich assay (b).

polymer substrate materials mentioned above. First, a base layer was polymerized as mentioned in the substrate preparation section. Then, a high-resolution photomask was placed in contact with argon-purged, monomeric matrix solution. The thickness was adjusted to 300 μm prior to collimated flood exposure, leading to a spatially controlled device layer atop the previous. Upon completion of exposure, unreacted monomer was removed via a methanol wash, and the polymerized trenches were filled with wax to prepare a level surface for further polymerization of sequential layers within the device.²⁹ Each 2-mm well was photografted for 900 s with either control PEG(375) monoacrylate monomer or specific antibody-PEG detection monomer for detection purposes. The microassay was removed from a polycarbonate base and void regions were cleared, resulting in a polymeric microassay, modified with surface chemistries for detection purposes. Photografted antibody was used to modify individual wells for selective detection of RAM-HRP and GAM-HRP antigen.

Microfluidic Immunosassay Surface Modification. GAR and DAG antibodies were acrylated and purified via the coupling procedure described for GAM-HRP. For this particular set of experiments, 2-mm-diameter wells on the second layer of the microassay were modified with 1.0 mg of GAR acrylate (includes mass of protein impurities) dissolved in 1 mL of PEG(375) for grafting, after exposure to UV light for 900 s. Similarly, a well was also modified with DAG antibody.

To verify device activity and specificity, a device with 2-mm-diameter wells grafted with GAR acrylate was tested. The device

was placed into a solution of 5.0 $\mu\text{g}/\text{mL}$ HRP-conjugated RAM antibody solution for binding with the surface-attached antibody. The device in solution was also incubated at 37 $^{\circ}\text{C}$ for 10 min. After this procedure, the wells were analyzed for surface activity, using previously described methods involving the reaction between Vector VIP and HRP. Similarly, detection of GAM-HRP was achieved sequentially. Note that the device assay time was extended to 30 min to amplify the detection intensity for better visualization of the smaller device wells.

Statistical and Data Analysis. Data sets were compared using single-factor ANOVA tests. P values of <0.05 were considered significant. All chromogenic intensity data was normalized to control samples where ELISA protocol or photografted antibody immunoassay protocol was used in the absence of antigen to eliminate concern related to false positives and nonspecific antibody activity.

RESULTS

Polymerizable Whole Antibodies. Acrylated, photopolymerizable whole antibodies (Figure 2a) provide a facile means for covalently attaching antibodies to polymer surfaces for the detection of specific antigens in a given analyte solution. Specifically, affinity purified, GAR, GAR-HRP, affinity purified, DAG, GAM-HRP, and anti-GLGN were acrylated using NHS/NH₂ conjugation, for detection purposes. Acrylation was verified via SDS-PAGE and a TNBS assay.³¹ Initially, DTT digestion of disulfide bridges was performed by boiling protein for 2 min, to yield heavy (50 kDa) and light (25 kDa) antibody fragments. SDS-

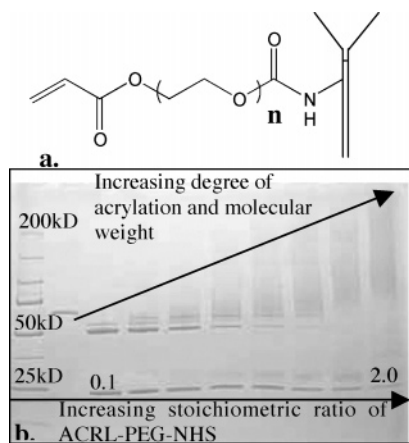


Figure 2. (a) Acrylated, PEG-conjugated antibody structure (only one acrylation shown here) where $n = 3400$. (b) SDS-PAGE result suggesting an increase in the degree of acrylation and molecular weight with increasing amounts of ACRL-PEG-NHS in the reaction stoichiometry. SDS-PAGE results indicate that more conjugation occurs in the heavy-chain portion of the antibody.

PAGE results illustrate an increase in molecular weight of both the heavy- and light-chain fragments after being reacted with increasing stoichiometries between 0.1 and 2.0 NHS/NH₂, to perform a range of conjugations. As evidenced in Figure 2b, the increase in acrylation is associated with an increased ACRL-PEG-NHS in the reaction stoichiometry. Although the SDS-PAGE results indicate conjugation on both heavy and light chains, the results do suggest a much higher degree of acrylation on the heavy-chain fragments of the antibody (bands starting at 50 kDa). This observation is significant and implies that less modification is occurring in the light-chain region of the antibody, leading to less interference with the antibody hypervariable region, associated with retention of antibody specificity. Moreover, under the aforementioned reaction conditions, the degree of acrylation, when using 1:1 NHS/NH₂ conjugation stoichiometry, was determined to be ~30%, as determined by a standard TNBS assay protocol³¹ monitoring the concentration of amine groups before and after modification.

Conjugated Antibody Specificity and Activity. Following acrylation, the antibody was coated on 96-well plates at varying concentrations to compare with detection activity of unmodified antibody. Figure 3a shows the detection of rabbit antigen using a standard ELISA protocol that gathers the results using both acrylated and unmodified primary antibodies. As illustrated by these results, only a slight decrease in the acrylated antibody activity was observed, when compared to ELISAs carried out with nonacrylated GAR using the same ELISA protocol. The activity was determined at varying concentrations of rabbit antigen (5.0×10^{-8} – 5.0×10^{-10} M), as shown. Further studies using standard ELISAs were completed to determine if the specificity of acrylated GAR antibodies was maintained for detection of nonspecific mouse antigen. Specificity results showed no statistically significant decrease in specificity when comparing modified and unmodified GAR antibodies.

Spatial Control of Antibody Grafts. Following verification of acrylated antibody activity and specificity, the ability to graft acrylated antibodies from a polymer surface via LRP photografting chemistry was demonstrated. GAR antibody was dissolved in PEG-

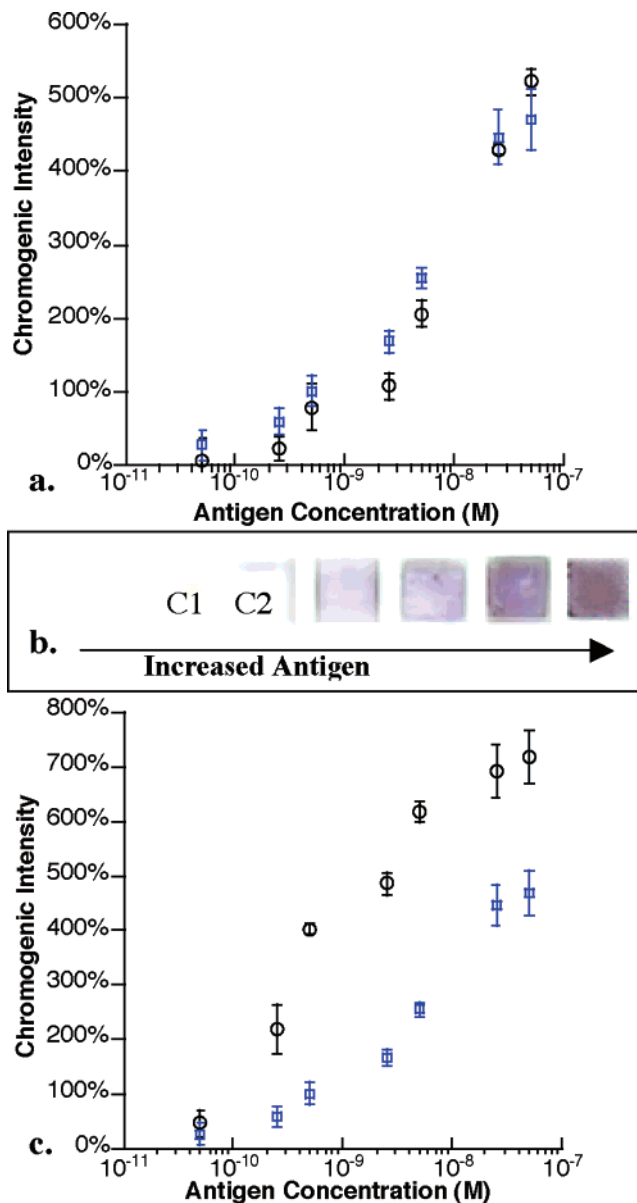


Figure 3. (a) Chromogenic intensity as a function of antigen concentration when using a standard ELISA technique that uses adsorbed, unmodified antibodies (\square) and adsorbed acrylated antibodies (\circ) for the detection of RAM antigen in PBS. This result demonstrates only a minor reduction in antibody activity after conjugation. (b) Photografted, square patterns of antibody-PEG acrylate monomer illustrate an increase in chromogenic intensity with increasing antigen concentration as compared control surfaces containing only PEG grafts (C1) and PEG grafts synthesized in the presence of nonacrylated antibody (C2). (c) Chromogenic intensity as a function of antigen concentration when detecting RAM using a standard ELISA technique (\square) and when using grafted antibody (\circ).

(375) monoacrylate at various concentrations (pM to nM) to formulate an initiator-free grafting solution. The incorporation of the PEG monomer leads to copolymer grafts where the pendant PEG groups serve to minimize nonspecific antigen interactions and further increase solvation and mobility of grafted detection chains. The DTC-mediated substrate grafting is achieved due to the incorporation of TED into the highly cross-linked polymeric base substrates. Using this LRP-based grafting scheme, acrylated antibody was covalently attached in 15 min, via UV photografting, to a polymeric

substrate surface. Figure 3b demonstrates the ability to control antibody grafting spatially. First, GAR was photografted and then exposed to rabbit antigen (5.0×10^{-11} – 5.0×10^{-8} M). The final substrate was assayed with a Vector VIP substrate kit to illustrate visually that individual surface-bound detection squares (5 mm \times 5 mm) are formed that maintain their active detection capabilities, after grafting. This result illustrates spatial resolution and an increase in chromogenic intensity with an increase in antigen concentration, as compared to control samples.

Further experiments confirmed that the antibody density in photografted chains was controlled by the photopolymerization time and the concentration of acrylated antibodies in the grafting solution. Significant increases in GAM–HRP graft density were achieved by increasing the UV exposure time, up to 15 min, which increased the surface concentration of recognized grafted antibody in a nearly linear fashion from 0.1 to 0.57 ng of antibody/cm². Also, by varying the concentration of acrylated antibody in the grafting solution, we were able to control the surface graft composition, as determined by chromogenic development of HRP using TMB. A consistent increase in active antibody surface composition was observed with increasing GAM–HRP concentration in PEG solution (0–0.10 mg of antibody/mL of solution) as compared to control PEG grafts in the presence of nonacrylated antibody (results not shown).

Accelerated Detection of Unlabeled Antigen. Minimizing Assay Time. The minimal reaction time for antibody (GAR) and a labeled antigen (RAM–HRP) was determined by exposing surface-bound antibody to a 5 μ g/mL solution of RAM–HRP in phosphate buffer solution (equal to 100 ng of antigen/square) for 2, 5, or 10 min. Bound antigen was detected colorimetrically by RAM–HRP reaction with a Vector VIP staining kit for 5 min. This reaction results in a surface-bound chromogenic response, where the amount of bound, HRP-labeled antigen is proportional to the intensity of the resulting purple color. The surface-bound chromogen (Vector VIP) was used to prevent chromogen diffusion when integrating this method onto a microfluidic device. Compared to a negative control of RAM–HRP reacted with a PEG-only graft, a 2-min reaction time resulted in a 25-fold increase in chromogenic response intensity, while at 5 min, the response was 50 times that of the control. The reaction achieved a maximum response after 5 min, as further reaction time did not significantly increase the response intensity.

Detection Sensitivity Range. Following identification of a suitable antibody–antigen reaction time, GAR-grafted antibody squares were also reacted with various concentrations of antigenic analyte to investigate the detection limits of this method. The detection limit is determined as an intensity that is statistically significant compared to that of a PEG-grafted square tested at the same conditions. GAR-grafted samples were prepared and reacted with a range of dilutions of RAM in PBS (5.0×10^{-11} – 5.0×10^{-8} M). After exposure to the secondary antibody, this procedure effectively forms an analyte “sandwich”, as antigen is bound to both the surface-tethered antibody and the HRP-labeled GAR. As shown in Figure 3c, the detection limit of this technique is less than 0.1 nM antigen in analyte solution and is comparable with ELISA results, even though detection was performed in \sim 15 min. Moreover, decreasing antigen concentration leads to a decrease in colorimetric response as expected ($p < 0.0001$). Interestingly,

due to less background on control samples, the chromogenic response for the sandwich assay performed with the grafted antibody method was typically much higher than that for the corresponding antigen concentration when using standard ELISA techniques.

Accelerated Detection of Unlabeled Glucagon Antigen. To demonstrate further the advantages of the rapid nature of this immunoassay technique, a sandwich assay was developed to allow for the detection of a more specific biological antigen. In this case, glucagon, a 29-amino acid peptide sequence that opposes the effects of insulin in gluconeogenesis and glycogenolysis, was detected using anti-GLGN antibody. With the rapid nature of our immunoassay method, GLGN was identified quickly (12–15 min) and specifically in PBS, 20% plasma and 20% whole blood biological environments, as shown in Figure 4a. As a comparison, an attempt to detect GLGN in plasma using standard ELISA techniques was made, and these results highlight the limitation of standard methods for detecting short-half-life molecules. Chromogenic intensities are insignificant throughout this range of GLGN dilutions, as shown in Figure 4b. The anti-GLGN grafted samples were prepared and reacted with a range of GLGN dilutions in PBS (1×10^{-5} – 1×10^{-13} M), showing sensitivity below the picomolar range. After 5 min of reaction time with Vector VIP, the detection limit of GLGN in PBS using the grafted antibody immunoassay was determined to be \sim 1.0×10^{-13} M GLGN antigen in PBS analyte solution. When GLGN was detected in whole blood- and plasma-containing systems, detection intensities were decreased by 66 and 53%, respectively; however, intensities remained significant and the detection limits remained comparable (pM) as noted in Figure 4a. The decrease associated with detection in plasma and whole blood environments is likely due to antibody interactions with the complex protein mixture found in plasma and whole blood, as compared to an ideal PBS buffer system.

Selective, Parallel Analyte Detection. Using the LRP-based grafting combined with a photolithographic technique,²⁹ a fully polymeric microfluidic device with antibody-grafted detection wells was constructed. For the design shown in Figure 5a, 2-mm-diameter wells incorporated on a device microassay were modified as follows: one with PEG(375) monoacrylate only, one with a mixture of DAG acrylate dissolved in PEG(375) monoacrylate for grafting, and one with GAR acrylate dissolved in PEG(375) monoacrylate for grafting. After proper washing, the antibody-modified wells of the microfluidic detection device were exposed to various antigens to demonstrate both the specificity of the covalently immobilized antibodies and the ability to use a microfluidic device to perform parallel detection of multiple analytes using this grafted antibody platform. A 0.1 mg/mL solution of GAM–HRP in PBS was exposed to all wells for 10 min and reacted with Vector VIP. This reaction yielded a positive response in well 3 as shown in Figure 5b, corresponding to the anti-goat antibody bound to well 3. It is important to note that cross-reactivity on nonspecific wells was negligible and did not lead to a false positive result. Following this reaction, the wells were exposed to a 0.1 mg/mL solution of RAM–HRP under the same reaction conditions. Well 2, containing an anti-rabbit antibody, developed a positive response due to specific detection of the rabbit-based antibody, as shown in Figure 5c. This result demonstrates the ability to detect multiple antigens in parallel wells on the same

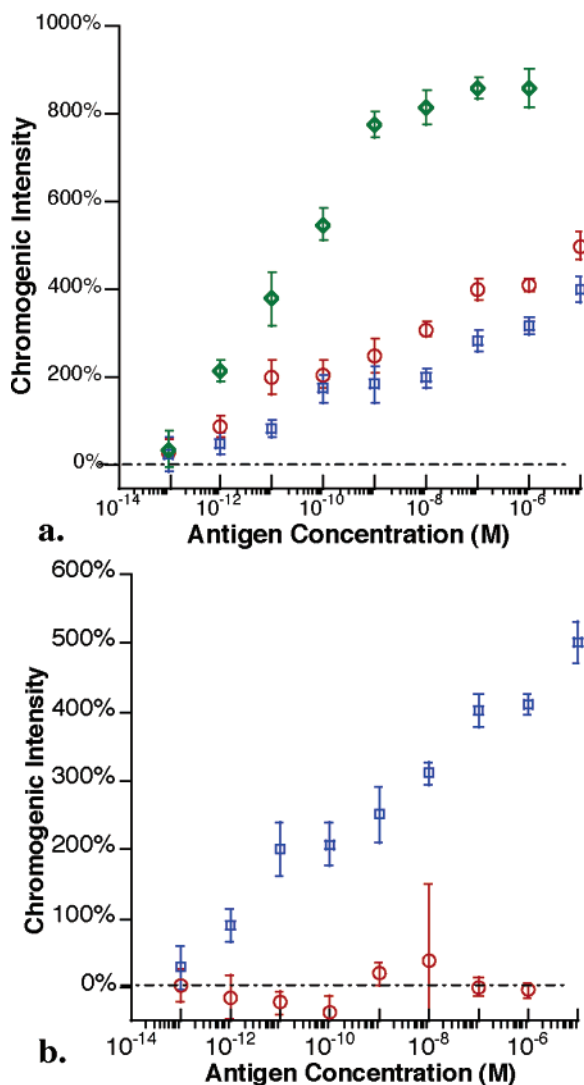


Figure 4. (a) Chromogenic intensity of Vector VIP (after 5 min) as a function of GLGN antigen concentration in PBS (◇), 20% plasma (○), and 20% whole blood (□) analyte. These results were determined using a grafted antibody assay, sandwiching unlabeled GLGN between GLGN-specific antibody tethers that were photografted for 900 s and HRP-labeled GLGN secondary antibody. All values are reported as a percentage intensity increase relative to control sample intensities in the absence of antigen (GLGN). The detection limits are shown as the point at which the sample intensity is not statistically different from that of a negative control (---) (1.0 pM for blood and plasma-containing samples and ~0.5 pM for the assay carried out in PBS). (b) Detection of GLGN in a 20% plasma environment, when using the grafted antibody immunoassay ($t = 15$ min.) (□) as compared to a standard ELISA technique ($t =$ hours) (○).

device. Further, the control well (PEG(375) only) that was not specific to the exposed antigens provided an insignificant chromogenic response when tested, illustrating the ability to prevent significant nonspecific protein adhesion to devices made by grafting with the PEG(375) acrylate.

DISCUSSION

To overcome limitations associated with common antibody immobilization techniques, acrylated antibodies were synthesized. The acrylate functionality is necessary as a photoreactive group

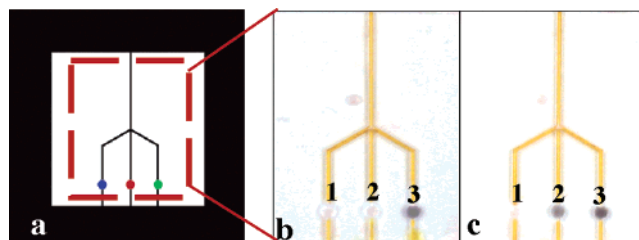


Figure 5. (a) Schematic of a three-well, parallel microfluidic detection device. The blue well is a control PEG acrylate-grafted well, the red is grafted with anti-goat antibody for detection of GAM-HRP, and the green is grafted with anti-rabbit antibody for detection of RAM-HRP. (b) Shows the chromogenic response using Vector VIP when well 3 detects RAM antigen. (c) The response when well 2 detects GAM antigen (post RAM testing). Note: Yellow dye has been added to device channels to improve the contrast.

that provides a means of covalently attaching antibodies from polymer surfaces or networks. Moreover, the functionalized antibodies are polymerizable through a UV-initiated mechanism in a spatially and temporally controlled fashion. When covalently attached in this fashion, the antibodies retain biological activity and specificity for rapid antigen detection purposes. Using acrylated antibodies in combination with classical photochemistry to design controlled surface architectures has additional advantages, complimented by our previous research.³² Through direct surface conjugation using acrylated antibodies, additional surface or antibody conjugation steps are removed to provide a single-step method to covalently attach spatially and temporally oriented antibodies (including combinations of antibodies³²) on polymer surfaces. Also, additional research could be performed to integrate our chemistry with existing platforms, using photografted molecules such as acrylated streptavidin, utilizing streptavidin's strong affinity toward biotin-functionalized antibodies.

Using the direct photografting approach, an immunoassay platform has been presented and exhibits picomolar sensitivity with specific utility for the detection of short half-life antigens or those that need to be detected in less than 20 min. The combined sensitivity and short assay time are the direct results of the ability to surface immobilize the antibodies on grafted chains of controlled length, chemistry, and composition, which eliminates time-consuming blocking steps and nonspecific protein interactions associated with standard assay techniques. The heightened activity of the antibodies on grafted chains is associated with the covalently polymerized PEG moieties that lead to highly solvated and mobile polymer grafts. This leads to increased antibody accessibility and minimizes issues associated with analyte diffusing to surfaces.

The LRP chemistry used to develop this platform is promising as a means to form both bulk and surface-initiated polymerized antibodies for diagnostic purposes. The method also facilitates simple integration of any antibody onto active polymer surfaces for detection purposes on fully polymeric microfluidic assays. Optimization of the tethered, active surface grafts is achieved through the use of rationally selected LRP chemistries, controlling the antibody surface density, orientation, and accessibility, hence decreasing the reaction time and increasing the antibody response potential. Additionally, this technique could be extended to facilitate covalent incorporation of growth factors, other proteins, and cell sensing moieties on or within polymeric matrixes, offering a significant impact on numerous biological applications.

Moreover, LRP photografting chemistry, coupled with photolithographic techniques, allows for construction of 3D microfluidic devices for highly efficient, parallel screening of various analyte systems that detect nano- to picomolar levels of analyte in as little as 12 min. This speed is made possible through enhanced mass transfer as well as the elimination of time-consuming blocking steps that are part of conventional assays. As illustrated in the figures, antibody grafted regions were specific and active toward the antigen of interest. Also, the rapid response time, when using grafted antibodies, is hypothesized to be a function of using PEG as a cografraft monomer with our antibody grafts. In this work, PEG simply blocks nonspecific antigen protein attachment over the detection times used and further increases chain solvation and mobility in a biologically relevant environment.

The assay platform that we have discussed in this research allows for either qualitative or quantitative evaluation of results without the requirement of expensive equipment, such as well-plate readers or UV-visible spectrophotometers. The results may be quantified simply by use of a digital scanner and readily

available, imaging software. Additionally, the ability to visualize results, combined with the portable size of microfluidic devices and the stability of covalently attached antibodies, indicates that this detection platform has significant implications in the development of portable assays. Finally, this platform provides an efficient means for on-the-spot screening and detection of various biological agents, including molecules that otherwise cannot be detected in a biologically complex environment such as plasma and whole blood.

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