A Rapid Antigen Detection Assay Using Photografted Whole Antibodies

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Introduction: Antibody surface immobilization techniques have had a significant impact on the detection of specific antigens for clinical diagnostics.[1,2] To date, most antigen detection assays (e.g., standard enzyme-linked immunosorbant assays) rely on monolayer formation or physisorption methods to immobilize antibodies to surfaces. However, that approach exhibits drawbacks associated with antibody coating stability and uniformity and can be heavily dependent on substrate properties, such as surface chemistry and roughness.[1,2] Thus, recent research has focused on methods to bind antibodies covalently to surfaces through conventional protein functional sites, such as amine and carboxy terminal groups, as well as antibody-specific thiol groups.[1,2,5] While these approaches reduce the possibility of antibody desorption, surface-bound antibodies and biomolecules often lose their activity and/or selectivity due to conformational and mobility restrictions or mass transfer limitations.[1,2] Furthermore, antibody activity is often lost due to a reduction of antigen binding sites resulting from the coupling process.

In this work, acrylated whole antibodies, termed antimers, were synthesized with the goal of establishing a unique polymerization method to immobilize antibodies to 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 clustering, which improves detection sensitivity and response time. In this research we demonstrate three significant milestones: the ability to polymerize whole antibodies as polymer grafts, which is integral in maintaining antibody activity and selectivity, the retention of antimer-antigen selectivity in a variety of biologically relevant analyte environments, and the application of a novel photopolymerization method, which allows patterning and fabrication of microfluidic assays based on antibody-antigen detection. These milestones have been demonstrated with a variety of antigens with varying molecular weight, biological stability and function.

Experimental Methods: Peroxidase-conjugated antibody was acrylated by first dissolving the antibody at 6 mg/ml in 50 mM sodium bicarbonate, pH 8.4, and reacting the antibody amine groups with monoacrylated poly (ethylene glycol) N-hydroxysuccinimide (ACRL-PEG-NHS, MW 3400) in a 1.1:1 molar ratio ($NHS:NH_2$). The reaction was allowed to proceed overnight at room temperature with shaking.

Antimer was covalently attached, via grafting (**Figure 1**), to the polymeric substrate surface using the living radical photopolymerization (LRP) surface chemistry, which utilizes photolabile dithiocarbamate groups.[3,4] A solution containing ~0.1 mg of labeled antimer (includes mass of any protein impurities) was mixed with 1 mL of PEG(375) monoacrylate solution for 10 minutes and purged with argon for 2 minutes prior to grafting. A patterned region of grafted antibody/PEG (375) acrylate was formed upon exposure to 45 mW/cm^2 intensity UV light for 900 seconds using photolithographic techniques.[4] The resultant pattern was washed in a solution of 100% ethanol for 24 hours followed by a 24-hour wash in deionized water.

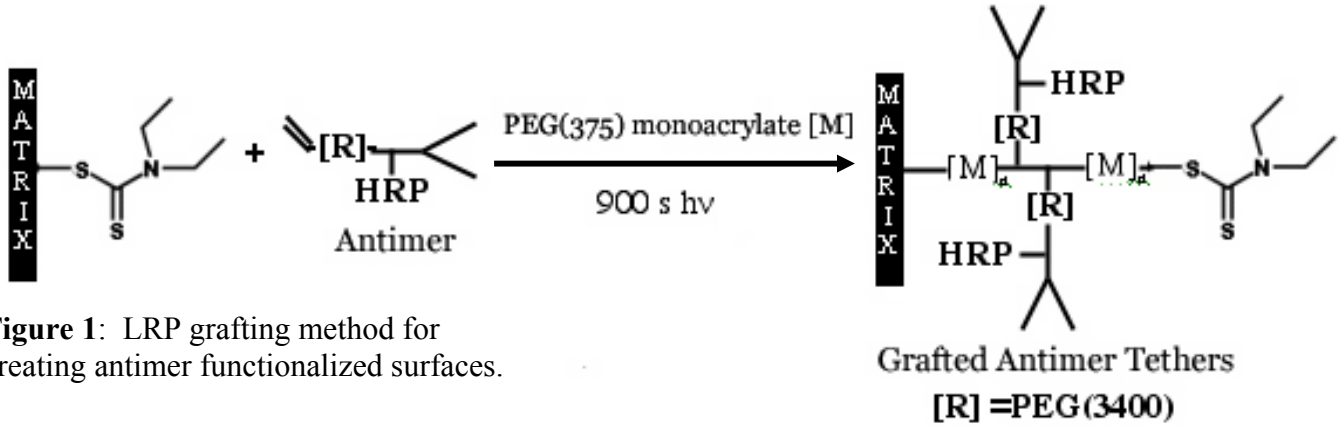

Figure 1: LRP grafting method for creating antimer functionalized surfaces.

After the antimer was grafted, a range of antigen dilutions was prepared in PBS. Antigen solutions in 20% whole blood in PBS and 20% plasma mixed with PBS are also being investigated and will be presented. These solutions were used immediately due to their stability in the presence of whole blood and plasma. Peroxidase-conjugated secondary antibody was added to all antigen dilutions at a concentration of 5 μg/ml and allowed to react with the antigen for 2 minutes prior to adding antigen to detection squares. After 5 minutes of reaction time at 37°C, devices were rinsed and colorimetrically analyzed using Vector VIP stain and grayscale analysis. Control samples consisted of antimer-grafted squares exposed to samples containing secondary antibody but in the absence of antigenic analyte.

Results and Discussion: Synthesis of acrylated, polymerizable antibodies is a useful means for covalently attaching antibodies to polymer surfaces for the detection of specific antigens in a given analyte solution. Specifically, affinity purified, goat anti-rabbit IgG (GAR), affinity purified, donkey anti-goat IgG (DAG), and anti-glucagon (GLGN) were acrylated for the detection purposes presented in this text. Acrylation was verified via light scattering and a TNBS assay[6]. The degree of acrylation was determined to be around 30%, using a standard TNBS assay protocol[6]. The average diameter of particles observed using light scattering was determined to be five times larger after UV exposure for 30 minutes in the presence of photoinitiator. This suggests an increase in average particle size upon UV-photopolymerization, due to chain polymerization of acrylated antimers present in solution.

Following verification of antimer acrylation and polymerizability, we demonstrated the ability to attach antimers successfully to a polymer surface via labeling. Further, we determined that the density of antimer in these grafts could be controlled via antimer solution concentration and photopolymerization time. Significant increases in labeled antimer graft density are achieved by increasing the UV exposure time, which increases the graft amount from 0-0.6 ng/cm^2 in a 900 second grafting period. In addition, a nearly linear increase in antibody surface composition (0.10-0.6 ng IgG/cm^2) was observed with increasing antimer concentration in PEG solution (0-0.10 mg antibody/mL solution) as compared to control PEG grafts in the presence of non-acrylated antibody.

Antimer utility was demonstrated in a novel immunoassay technique. A sandwich assay was developed to allow for the detection of rabbit anti-mouse (RAM) antigen. Anti-rabbit antimer grafted samples were prepared and reacted with a range of dilutions of RAM in PBS as shown in **Figure 2**. After reaction with Vector VIP, the detection limit of RAM in PBS using this rapid immunoassay was determined to be less than 0.033 μg/cm^2 antigen in PBS analyte solution. This is a positive result, but further research, dealing with the detection of a biologically relevant species in whole blood is being pursued.

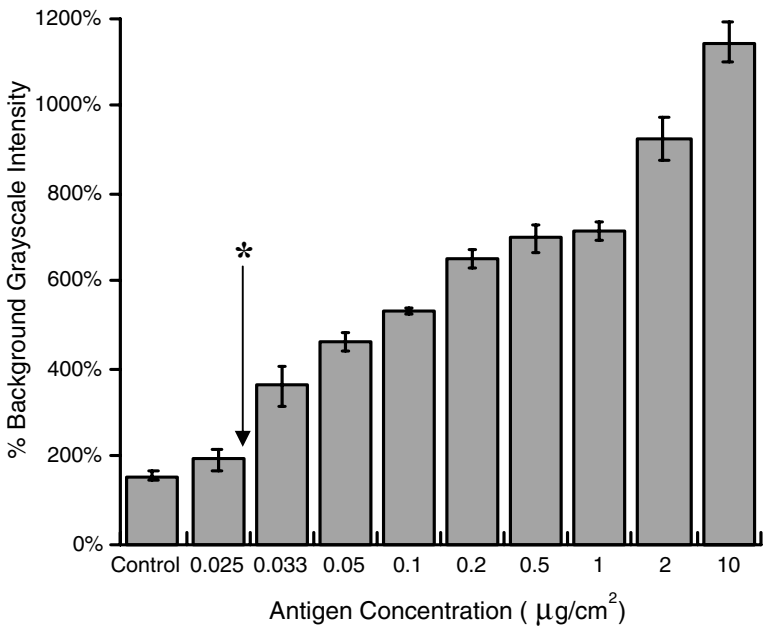

Figure 2: Chromogenic intensity of Vector VIP (after 5 minutes) as a function of RAM antigen concentration (0-50 μg/mL) for an assay sandwiching unlabeled RAM between GAR antimer tethers that were photografted for 900 seconds and HRP-labeled secondary antibody. All values are reported as percentage compared to polymer control substrate grayscale intensity. Detection limits are pointed out (*) with arrows and signify when the background intensities approach that of the control sample.

Using LRP-based grafting combined with our photolithographic technique[4], a 3D microfluidic device with antibody-grafted detection wells was constructed. This device was created for the purpose of demonstrating our ability to detect multiple analytes simultaneously, also confirming that the immobilized antimers have retained their specificity for the appropriate antigens following grafting. For the design shown in **Figure 3**, 2 mm diameter wells incorporated on a microassay device were modified with antimer. After proper swelling and cleaning, the 2 mm diameter, antimer-grafted wells were utilized for detection purposes as explained in **Figure 3**.

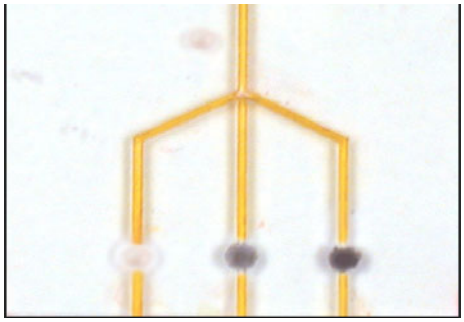

Figure 3: A parallel microfluidic detection device demonstrating positive detection of two specific antigens in parallel. The left well is a negative control well, consisting of a PEG only graft, in the absence of antimer.

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