Influence of Ancillary Binding and Nonspecific Adsorption on Bioresponsive Hydrogel Microlenses

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We report investigations of specific and nonspecific adsorption effects on bioresponsive hydrogel microlenses to better understand their utility and potential advantages for biosensing. Bioresponsive microgels were prepared from stimuli-responsive poly(*N*-isopropylacrylamide-*co*-acrylic acid) (pNIPAM-*co*-AAc) microgels after functionalization with both biotin and ABP (as a photoaffinity label) via carbodiimide chemistry. Bioresponsive hydrogel microlenses were then constructed from the microgels via Coulombic assembly of the anionic microgels on a positively charged, silane-modified, glass substrate. Specific and nonspecific protein binding on the hydrogel microlenses was studied by monitoring the optical properties using brightfield and fluorescence optical microscopies. The bioresponsivity, as determined by changes in the microlensing power, is strongly coupled to the formation of cross-links via ligand−protein and/or antigen−antibody binding. However, the microlensing phenomenon and the intrinsic bioresponsivity of the hydrogels are completely insensitive to simple adsorption via nonspecific protein binding from reconstituted human serum. These results suggest that the hydrogel microlens construct may be a good candidate for a wide range of applications in which the bioresponsive material would be required to operate in complex biological media.

Introduction

Numerous investigations on bioresponsive hydrogels, which undergo physicochemical changes in response to a biological stimulus, have been conducted over the past decade due to their potential applications in drug delivery, bioassays/biosensors, tissue engineering, and bio-mimic scaffolds.[1−19] Conventional responsive hydrogels are often thermoresponsive due to a reversible phase separation behavior at the lower critical solution temperature (LCST) or upper critical solution temperature (UCST) of the polymeric gels.[20−25] Other responsive gels, which may respond to pH, ionic strength, photon flux, and electricity, have also been investigated extensively.[26−34] Recent efforts to make gels bioresponsive have shown significant success in the fields of cell targeting, new biosensors, and biocompatible materials.[5,9−12,35] For instance, bioassay/biosensing devices have been developed wherein the gel swelling/deswelling is coupled to a change in the number of hydrogel cross-links, where the cross-linking is formed and/or deformed by biological binding events, such as those formed from antibody−antigen or protein−ligand binding pairs.[7,10−12]

Our group has recently shown that responsive hydrogel microparticles can be used to prepare tunable microlenses that respond to temperature, pH, and photon flux, where the focal lengths of the hydrogel microlenses are changed by hydrogel swelling/deswelling transitions.[31,36] In light of the results, we have successfully coupled the swelling of a hydrogel microlens to antibody−antigen association/dissociation equilibria, thereby forming bioresponsive optical elements.[10,11] In our approach, excess cross-linking at a microlens surface is triggered via the association of covalently tethered antibody−antigen pairs, resulting in hydrogel deswelling at the periphery of the microparticle. By introducing free antigen to the surrounding medium, the hydrogel-bound antigen is displaced by free antigen, which results in a decrease in the degree of hydrogel cross-linking at the microlens outer surface, thus causing reswelling of the gel periphery. In previous studies, we have shown that the thermodynamics of hydrogel swelling are coupled to the thermodynamics of the biological binding events, and thereby suggested that a general platform for bioresponsive materials in response to diverse biological events can be prepared. Furthermore, the surface localized swelling/deswelling strategy of the bioresponsive microgels is favorable for the rapid and reversible responsivity of the gel structure mainly due to the short diffusion distance and less chance of entrapment of the analyte throughout the hydrogel network.

In this Article, we report the investigation on the intrinsic selectivity of the bioresponsive microgel construct to a target molecule. For this study, specific (but ancillary) and nonspecific binding effects on the bioresponsive microlenses were evaluated by observing the optical properties of the microlenses when they are exposed to a solution of a secondary IgG (for specific, ancillary binding) and a solution of antigen dissolved in reconstituted human serum (for nonspecific binding). These experiments enable a better understanding of the utility and potential advantages/limitations of the bioresponsive microlens construct not only for label-free biosensing but also for new stimuli-responsive biomaterials. We find that the technique described here does not suffer from false signals by nonspecific adsorption, mainly due to the fact that the microlensing is predominantly modulated by antibody:antigen (and/or protein:ligand) cross-linking rather than by simple protein adsorption.

Materials and Methods

Materials. All reagents were purchased from Sigma-Aldrich unless otherwise specified. The monomer *N*-isopropylacrylamide (NIPAm) was recrystallized using hexanes (J.T. Baker) prior to use. The cross-linker

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N,*N*′-methylene(bisacrylamide) (BIS) and the initiator ammonium persulfate (APS) were used without further purification. Acrylic acid (AAc) was distilled under reduced pressure. The glass coverslips used as substrates were 24 × 50 mm Fisher Finest brand cover glass. The cationic silane 3-aminopropyltrimethoxysilane (APTMS) was used for the functionalization of the glass substrates. Absolute (200 proof) and 95% ethanol were used for various purposes in this investigation. The water-soluble carbodiimide 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC) and biotin hydrazide were purchased from Pierce. Dimethyl sulfoxide (DMSO) was obtained from J.T. Baker. Polyclonal anti-biotin (raised in goat) was purchased from Sigma-Aldrich. Unlabeled avidin, fluorescent avidin conjugate (Texas red), fluorescent rabbit anti-goat IgG (Alexa fluor 594), and biotin-4-fluoresein were obtained from Molecular Probes. Water was distilled and then deionized (DI) to a resistance of at least 18 MΩ (Barnstead Thermolyne E-Pure system) and then filtered through a 0.2 μm filter to remove particulate matter before use. For printing the projection pattern, 3M transparency film and a Hewlett-Packard LaserJet 4000N printer were used.

Methods. *(1) Microgel Synthesis and Functionalization.* Bioresponsive microgels (~1.5 μm diameter) were prepared by free-radical precipitation polymerization of monomer NIPAm, cross-linker BIS, and co-monomer acrylic acid. The reactant mixture was composed of a total monomer concentration of 300 mM with a molar composition of 89.4% NIPAm and 0.5% BIS and was made by dissolving the monomers in 100 mL of deionized water. In a 250 mL three-neck round-bottom flask, filtered monomer solution was purged with N_2 and heated to 70 °C. The reaction solution was maintained at the same temperature throughout the synthesis. After 60 min, co-monomers AAc (10%) and 4-acrylamido-fluorescein (0.1%) were added to the reaction mixture followed by the addition of 1 mL of 6.13 mM APS to initiate the polymerization process. The copolymerization was allowed to proceed for 4 h at 70 °C under N_2. The resultant colloidal dispersion was dialyzed against water for ~2 weeks with the water being changed twice per day, using 10 000 MW cutoff dialysis tubing (VWR).

Biotinylation of the AAc carboxyl groups was carried out by first diluting the pNIPAm-AAc microgel solution 10-fold in a 2-[*N*-morpholino]ethanesulfonic acid (MES) buffer (pH 4.7). To 1 mL of dilute microgel solution was added biotin hydrazide (3.8 mg dissolved in 0.5 mL of DMSO) followed by the addition of 15 mg of EDC. The reaction was kept overnight at 4 °C while stirring. Unreacted biotin hydrazide was removed by several cycles of centrifugation followed by resuspension of the microgel pellet in phosphate buffered saline (PBS) (pH 7.5).

To covalently couple the antigen-bound antibody to the microlenses to form a reversible cross-link, a photoaffinity approach is used. Aminobenzophenone (ABP) is coupled to the particles and then can be used to affect photoattachment of the antibodies once they have assembled (via antigen binding) on the microlens surface.[11] Without this step, the antibodies would simply diffuse off the microlens surface once they were displaced with free antigen. We have also shown previously that the ABP present on the microlenses that is confined between the microlens and the silanized glass substrate following deposition can be used to covalently couple the microlenses to the glass.[11] This provides for a more robust sensor design.

For the modification of the biotinylated pNIPAm-AAc particles with 4-aminobenzophenone (ABP), the biotinylated microgel particle solution (1 mL) was centrifuged five times, and the resulting white pellet was redispersed in 700 μL of DMSO. To this was added 150 μL each of 0.01 M ABP and 0.01 M DCC solution in DMSO. The reaction solution was stirred overnight at room temperature in the dark. Addition of 0.5 mL of deionized water to the reaction solution resulted in a white solid precipitate of *N*,*N*′-dicyclohexylurea, which was filtered off. The resultant filtrate was centrifuged at 14 000 RPM for 15 min, and the pellet was redispersed in DMSO followed by four additional centrifugation cycles and redispersion in PBS buffer (pH 7.5). Note that the overall synthetic scheme employs equimolar amounts of biotin and ABP for coupling with the carboxyl groups, and the overall stoichiometry between biotin+ABP and AAc is 1:1. However, the reaction efficiency of the carbodiimide coupling is <100%; hence the unreacted carboxyl groups can be further used for binding to the cationic glass substrate during the initial assembly step.

(2) Bioresponsive Hydrogel Microlens Substrate Preparation. To remove any organic residuals from the surface, glass coverslips were treated in an Ar plasma (Harrick Scientific) for 30 min. Plasma treatment was followed by immersion of the glass substrates in an ethanolic (absolute ethanol) 1% APTMS solution for ~2 h, after which they were removed from the solution and rinsed several times with 95% ethanol. These silane-functionalized glass substrates were stored in 95% ethanol for no longer than 5 days prior to use. Prior to microgel assembly, the substrates were rinsed with DI water and gently dried in a stream of N_2 gas.

Two different microlens assemblies were used to study the protein binding effects. Biotin-modified microgel assemblies were formed by exposing a silane-modified substrate to a biotin-modified microgel solution in 10 mM PBS buffer (pH 7.5). After a 5 min exposure, the substrate was immersed in DI water for 2 h, rinsed with DI water, and dried with N_2 gas to leave behind microgels that are strongly attached to the substrate by Coulombic interactions. The second type of substrate consists of the biotin+ABP-modified microgels. Silane-functionalized glass substrate was exposed to an aqueous biotin+ABP-modified microgel solution buffered by 10 mM PBS buffer (pH 7.5). After 30 min, the substrate was rinsed with DI water and dried with N_2 gas to leave behind microgels strongly attached to the substrate. As an internal reference, nonfunctionalized microgels were also attached to each substrate in a similar way.

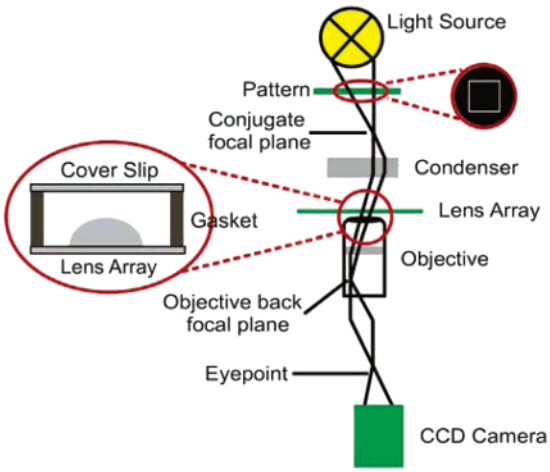
A microlens array/silicone gasket/coverslip sandwich assembly was then prepared using the microgel-functionalized glass slides. A solution of polyclonal anti-biotin diluted with pH 7.5 PBS was introduced into the void space of the microlens array/silicone gasket/coverslip sandwich assembly. After 3 h of antibody incubation, the substrate was rinsed and the medium was replaced with pH 7.5 PBS. The biotin/ABP-modified microgel assemblies were UV irradiated using a 100 W longwave UV lamp for 30 min while cooling the coverslip on an ice bath.[37] This process photoligates the antigen-bound antibody to the microgel network. For microscopic investigations of microlens response to competitive antigen:antibody binding, various concentrations (100 μL aliquots) of biotin, Alexa fluor-labeled avidin, IgG, biocytin, and antibiotin buffered in 10 mM PBS were introduced into the void space of the assembly.

(3) Microscopy. Brightfield transmission and differential interference contrast (DIC) optical microscopies were used to study the changes in the optical properties of substrate supported hydrogel microlenses. An Olympus IX70 inverted microscope equipped with a high numerical aperture, oil immersion 100× objective (NA = 1.30) was used for all microscopies reported here. Images were captured using a black/white CCD camera (PixelFly, Cooke Corp.). The microscope setup used in this experiment is shown in Scheme 1. The image being projected in these studies is a white square, positioned as indicated in the scheme. In this setup, we focus the microscope such that the square is in focus in the microlens "off" state, and when the microlens focal length decreases (microlens "on" state), the square image becomes minified to the center of the microlens, thus producing what appears to be a bright spot at the center of the microlens.

Results and Discussion

The main purpose of this study is to examine specific and nonspecific adsorption effects on bioresponsive hydrogel microlenses to elucidate the utility and potential advantages of this bioresponsive material. To develop bioresponsive materials for applications in complex biological media, selective binding between the analyte and the sensing material is required, while any nonspecific binding events must not perturb the sensor response. To investigate these effects, pNIPAM-*co*-AAc hy-

Scheme 1. Inverted Light Microscopy Setup Used for Aqueous Phase Imaging Experiments[a]



[a] The microlenses at the imaging plane move the objective back focal plane to the eyepoint, thus bringing the square pattern near the source into focus.

drogel microparticles were first functionalized with biotin via EDC coupling (Scheme 2, route a) or with biotin+ABP via EDC and DCC coupling (Scheme 2, route b). For the direct protein binding study (route a), the biotinylated hydrogels are used to prepare microlenses as described in previous studies.[10] Also, reversibly bioresponsive hydrogel microlenses (route b) are prepared by using biotin+ABP-functionalized hydrogels via anti-biotin incubation followed by photoligation. The reversible microlenses are then exposed to antigen spiked reconstituted human serum for the evaluation of nonspecific binding effects. Exposure to solutions of a secondary IgG allows for the interrogation of specific/ancillary binding effects.

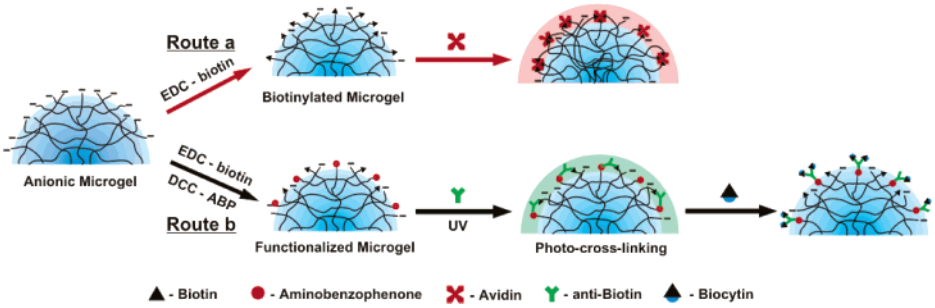
Specific binding of Texas red-labeled avidin to biotinylated hydrogel microlenses was investigated using fluorescence microscopy, as shown in Figure 1. The microgels are fluorescently labeled by fluorescein for visualization; both biotinylated and non-biotinylated microgels were adsorbed to the substrate to provide an internal negative control. Thus, the relative degrees of red (avidin) and green (microgel) emission allow for determination of avidin binding. In Figure 1, red fluorescence is observed at the outer rim of the biotinylated microgels when they are exposed to various concentrations (10 nM to 1 μM) of avidin solution (Figure 1b−d). Obviously, no red emission is observed in PBS buffer only (Figure 1a). On the other hand, nonspecific adsorption of avidin to the non-biotinylated microgels appears to be negligible over the same concentration range. Note that both types of microgel yield the same results under the same experimental conditions when they are separately assembled on substrates as homogeneous populations (data not shown).

To investigate the reliability of the bioresponsive microgels in complex media, hydrogel microlenses were prepared by route b in Scheme 2 and then are exposed to various antigen concentrations spiked into ImmunoPure Normal Human Serum (Figure 2). As illustrated in our previous studies,[10,11] the focusing power of the microlenses can be tuned by swelling or deswelling of the microgels. This swelling is coupled to the number of noncovalent cross-links formed by biotin:anti-biotin pairs at the microlens exterior. We characterize the projected images through the microlenses as being "on" or "off" as a function of the swelling state; the optical setup used to form these images is described in the experimental section and is shown in Scheme 1. The data in Figure 2 illustrate that the microlens-projected images are easily resolvable in human serum; the "on" and "off" states are easily identified, and the state is dependent on the biocytin concentration. In this figure, the biocytin concentration increases along the row (as indicated), and the two different rows correspond to two different concentrations of antibody used to prepare the microlens. We find from these data that the microlens sensitivity is the same as that which we have previously reported for experiments performed in buffer.[11] These results suggest that the bioresponsivity of the hydrogel microlenses is highly resistant to interference due to nonspecific binding and that the optical setup is operational even when the medium refractive index is increased due to a high background protein concentration.

We have also observed that the displacement-based sensor design yields microlenses that are tolerant to moderate levels of a secondary specific binding event. Figure 3a shows the DIC and image projection views of a single bioresponsive microlens in PBS. A solution of anti-goat IgG (raised in rabbit and labeled with Alexa fluor 594) was then introduced. This antibody should bind to the tethered antibody (which is a goat IgG), but should do so by binding to the non-paratope regions and should therefore not appreciably disrupt the antigen:antibody (biotin:anti-biotin) interactions. Indeed, we find that there is no discernible change in the microlens appearance or microlens-projected images (Figure 3b) under secondary IgG binding conditions. Fluorescence microscopy reveals that the secondary antibody is indeed bound to the particle periphery. Subsequently, the microlenses were exposed to a 100 μM solution of biotin-4-fluorescein (PBS buffer). Under these conditions, the microlens switches from the "on" to the "off" state, as seen in both the DIC and the image projection modes (Figure 3c). Fluorescence microscopy confirms that the secondary antibody is still bound to the microlens-tethered anti-biotin, while the biotin:anti-biotin binding at the microlens surface is disrupted by displacement. Originally, we had hoped to also visualize the green emission from biotin-4-fluoresein. However, it is not observed under these conditions either due to a low bound antigen concentration, quenching by Texas red, or photobleaching of fluorescein.

The results presented above are interesting if one considers the fact that the secondary IgG should be able to form additional cross-links at microlens periphery by binding 2 equiv of the microlens-tethered anti-biotin. Thus, while the secondary IgG binding might not induce displacement of the biotin:anti-biotin interactions, it might form additional anti-biotin:IgG:antibiotin associations, which would increase the cross-link density at the microlens periphery. To investigate this possibility, we employed the same experimental conditions as those described for Figure 3 with increasing concentrations of the secondary IgG; the data obtained are compiled in Table 1. These data show that the microlens biosensing can be perturbed by the formation of these secondary cross-links. For example, if the microlenses are exposed to a 0.5 or 1 μM solution of the secondary IgG and then exposed to a 1 mM biocytin solution, the microlenses remain in the "on" state. Fluorescence microscopy shows that the secondary IgG remains bound, as was seen in Figure 3. The corresponding data from Figure 3 (this time performed with biocytin as the antigen) are included in column 4 of the table for comparison; at only 0.1 μM secondary antibody, the addition of 1 mM biocytin is sufficient to switch the microlens state. These results suggest that at higher concentrations of secondary IgG, ancillary cross-links can be formed (anti-biotin:IgG:antibiotin interactions) at the microlens periphery, which then lock the microlens in the "on" state. Biocytin-induced displace-

Scheme 2. General Concept for Protein Binding Using Bioresponsive Hydrogel Microlenses[a]



[a] Route a: The pNIPAm-AAc microgels are biotinylated via EDC coupling and then exposed to fluorescently labeled avidin to show biotin-avidin binding. Route b: The microgels are functionalized with biotin and ABP via EDC and DCC coupling, respectively. These microgels are then incubated with anti-biotin followed by photoligation via UV irradiation, and then exposed to target molecules to reversibly disrupt the microgel-bound antibody−antigen interactions.

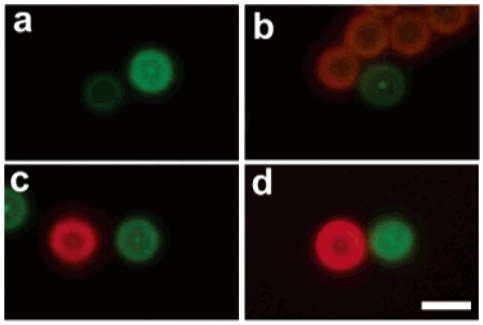


Figure 1. Selective binding of avidin to biotinylated hydrogel microlenses in (a) no avidin (PBS buffer only), (b) 10 nM avidin, (c) 100 nM avidin, and (d) 1 μM avidin dissolved in 10 mM PBS. Note that avidin (red) is labeled with Texas red, and the microgel (green) is labeled with 4-acrylamidofluorescein. The fluorescence microscopy images reveal that the biotin−avidin binding is only observed on the biotinylated microgels. Incubation time = 1 h. Incubation temperature = 22 °C. The scale bar is 2 μm.

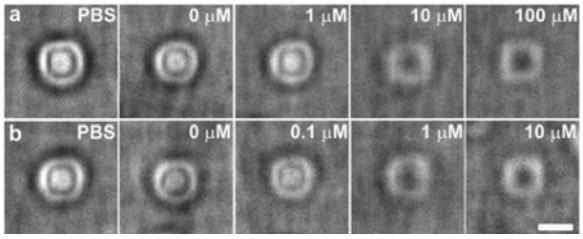


Figure 2. Sensitivity of hydrogel microlenses to various antigen concentrations in normal human serum. The hydrogel microlenses were incubated in (a) 1 μM anti-biotin solution, and (b) 0.3 μM anti-biotin solutions, followed by photo-cross-linking to render them reversibly bioresponsive. Normal human serum (protein concentration, 60 mg/mL) was used for the negative control experiment (second column). PBS buffer solution was used for a reference medium in the experiment (first column). The biocytin concentrations in the human serum are indicated at the top right of each column. Incubation time = 3 h. Incubation temperature = 22 °C. The scale bar is 2 μm.

ment of the biotin:anti-biotin cross-links is insufficient to swell the microlens to the "off" state in the presence of these ancillary cross-links. These results indicate that, while the displacement-based bioresponsive microlens construct can be very insensitive to nonspecific binding, multivalent secondary binding events can perturb the sensor and may result in false negatives in an analytical application.

Conclusions

We have demonstrated that the responsive behavior of the bioresponsive hydrogel microlenses is exclusively triggered by

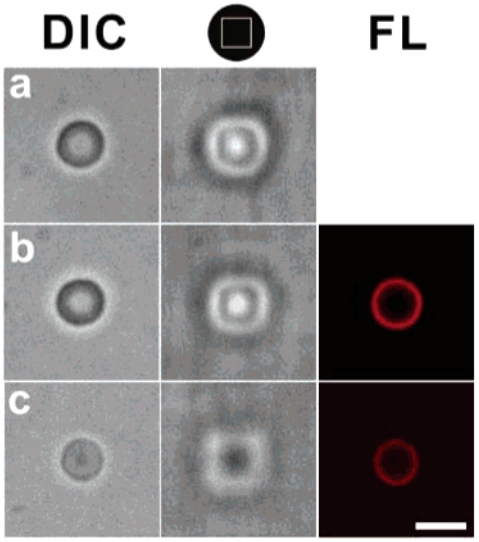


Figure 3. Effects of secondary specific adsorption on the responsivity of microlenses prepared with 1 μM anti-biotin: (left column) DIC image of the hydrogel microlens, (center column) projected square pattern images through the hydrogel microlens, and (right column) fluorescence microscopy images in: (a) 10 mM PBS buffer pH 7.5, (b) 0.1 μM rabbit anti-goat IgG conjugated with Alexa Fluor 594, and (c) 100 μM biotin-4-fluorescein. Incubation time = 3 h. Incubation temperature = 22 °C. The scale bar is 2 μm.

Table 1. Effects of Secondary Specific Adsorption on Bioresponsive Microlenses[a]

[anti-biotin] (DIC[b])	1 μM (on)	1 μM (on)	1 μM (on)
[anti-IgG] (DIC,[b] FL[c])	1 μM (on, bound)	0.5 μM (on, bound)	0.1 μM (on, bound)
[biocytin] (DIC,[b] FL[c])	1 mM (on, bound)	1 mM (on, bound)	1 mM (off, bound)

[a] For all experiments: incubation time = 3 h; incubation temperature = 22 °C. [b] Indicates the "on" or "off" state as determined by DIC microscopy. [c] Indicates that the anti-IgG is bound, as determined by fluorescence microscopy.

the formation/disruption of cross-links via ligand:protein or antigen:antibody interactions. We also show that the hydrogel microlensing is highly resistant to interference by simple protein adsorption; either nonspecific adsorption does not occur in human serum, or such adsorption does not perturb the sensor function. Furthermore, the control experiment with a secondary IgG confirms that the biosensing/bioassay function is strongly dependent on the cross-link formation. Thus, we have illustrated both the potential power of the construct (insensitivity to

nonspecific adsorption) and its limitations (strong multivalent secondary binding events) as a generalized biosensor construct.

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