Electronic Supplementary Material (ESI) for Analyst.

Ultrasensitive Carbohydrate-Peptide SPR Imaging Microarray for Diagnosing IgE-Mediated Peanut Allergy

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Supporting Information

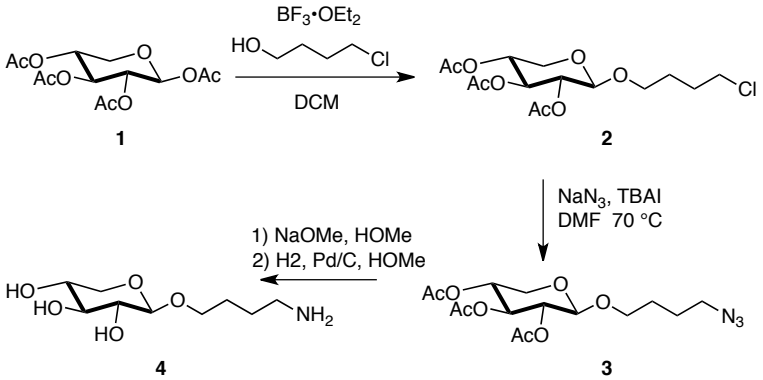
Materials and methods.

Human IgE mixture was obtained from Thermo Scientific (Clone HE1, Product no # DIA-HE1-01) This product contains purified human IgEs with kappa light chains produced in vitro from a monoclonal hybridoma cell line. Original material was obtained from healthy donors that tested negative against HIV, HCV and hepatitis B. The purity of IgEs is >98%, tested by SDS-PAGE by the vendor.

Affinity-purified chicken anti-peanut protein (IgY) was from Gallus Immunotech Inc. NC, USA. Monoclonal Anti-Human IgE (Ab_1) (Product no# I6510) and polyclonal ε- chain specific anti-human IgE (Ab_2) (Product no# I6284) were from Sigma Aldrich (MO, USA).

Immunoreagents were dissolved in pH 7.2 phosphate buffer saline (PBS, 0.01 M phosphate, 0.14 M NaCl, 2.7 mM KCl) unless otherwise noted. Reagents for all SPR studies were passed through a 0.2 μm filter and degassed before use. 1-ethyl-3-(3-dimethylamino-propyl) carbodiimide (EDC) , 0.1 M N-hydroxysuccinimide (NHS), and N-hydroxysulfosuccinimide (NHSS) were from Sigma Aldrich. Solutions are prepared fresh right before use.

Synthesis of β-xyloside



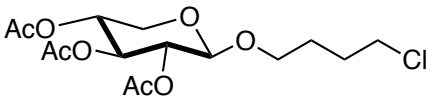
Scheme S1. Synthesis of β-xyloside with terminal butyl amine group (**4).**

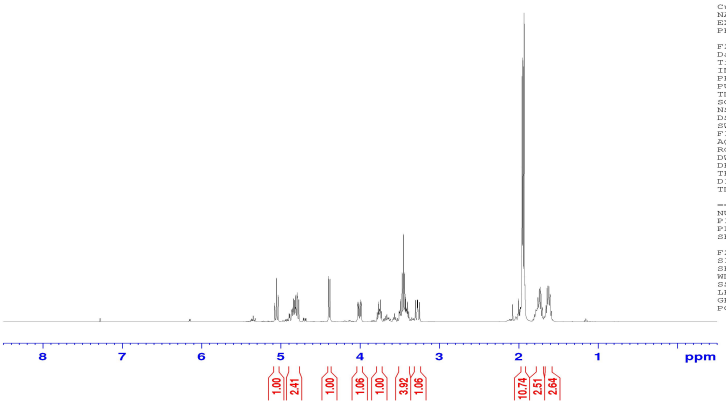
4-Chlorobutyl 3,4,5-tri-*O*-acetyl-β-D-xyloside (2). 1,2,3,4-Tri-*O*-acetyl xylose **1** (0.500 g, 1.57mmol) was dissolved in dry DCM (15 mL). To this solution was added 187 μL 4-chlorobutanol (1.88 mmol, 1.2 eq) and 300 μL BF_3•OEt_2 (2.36 mmol, 1.5 eq). The mixture was stirred at room temperature for 4h under an atmosphere of N_2. The reaction was then quenched by addition of 0.500 g solid K_2CO_3. The quenched reaction was allowed to stir for an additional 30 min, then the mixture was washed (2 x 15 mL) with water. The organic layer was dried by the addition of solid Na_2SO_4, the solution was filtered and the solvent was removed from the filtrate under reduced pressure. The residue was purified by column chromatography (3:1

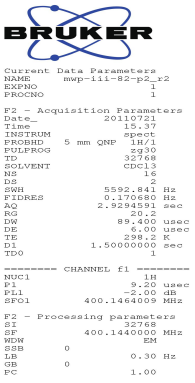
Hex:EtOAc) to give 2 as a clear, colorless oil (0.263 g, 48%). ^{1}H ($CDCl_3$) δ 400 MHz 5.06 (dd, *J* = 8.7, 8.7 Hz, 1H), 4.90-4.77 (m, 2H), 4.39 (d, *J* = 6.9 Hz, 1H), 4.01 (dd, *J* = 11.8, 5.1 Hz, 1H), 3.76 (ddd, *J* = 12.0, 6.0, 6.0 Hz, 1H), 3.51-3.39 (m, 3H), 3.28 (dd, J = 11.8, 8.9 Hz, 1H), 1.96 (s, 3H), 1.95 (s, 3H), 1.93 (s, 3H) 1.77-1.71 (m, 2H), 1.66-1.59 (m, 2H); ^{13}C ($CDCl_3$) δ 100 MHz 169.9, 169.7, 169.3, 100.6, 71.3, 70.7, 68.8, 68.5, 61.9, 44.6, 29.1, 26.7, 20.6 (2).

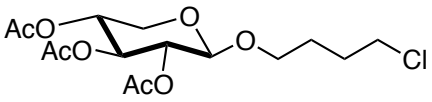
4-Azidobutyl 3,4,5-tri-*O*-acetyl-β-D-xyloside (3). Into a round bottom flask were added compound **2** (0.263 g, 0.72 mmol) and tetra-*N*-butylammonium iodide (0.026 g, 0.07 mmol) and 5 mL dry DMF. Solid NaN_3 (0.280 g, 4.32 mmol) was added and the mixture was heated to 70 °C under N_2 for 18 h. After, the reaction was allowed to cool to room temperature and the solvent was removed under reduced pressure. The mixture was then redissolved in EtOAc (30 mL) and washed with (2 x 15 mL) with water and brine (1 x 15 mL). The organic layer was dried by the addition of solid Na_2SO_4, the solution was filtered and the solvent was removed under reduced pressure to give 0.216 g of **3** as a clear, pale orange oil (81%). ^{1}H ($CDCl_3$) δ 400 MHz 5.10 (dd, J = 8.6, 8.6 Hz, 1H), 4.94-4.82 (m, 2H), 4.42 (d, J = 6.8 Hz, 1H), 4.05 (m, 1H), 3.81-3.76 (m, 1H), 3.47-3.42 (m, 1H), 3.31 (dd, J = 11.9, 9.0 Hz, 1H), 3.25-3.22 (m, 2H), 2.00 (s, 3H), 1.99 (s, 3H), 1.97 (s, 3H), 1.62-1.59 (m, 4H); ^{13}C ($CDCl_3$) δ 100 MHz 170.0, 169.8, 169.4, 100.6, 71.5, 70.8, 68.9, 68.8, 62.0, 51.1, 26.6, 25.5, 20.7(2).

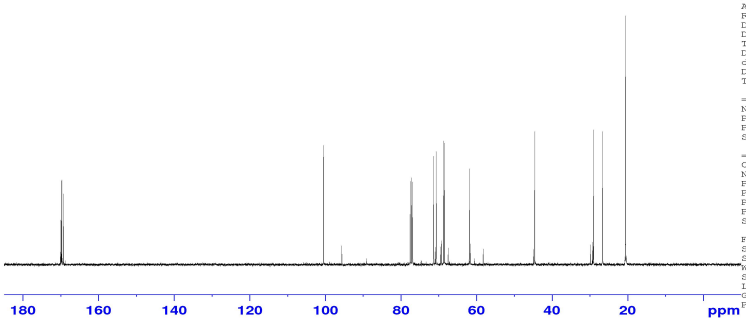
4-Aminobutyl β-D-xyloside (4). Azidobuty β-xyloside **3** (0.216g, 0.59 mmol) was dissolved in 600 µL of a 0.1 N NaOMe in MeOH solution. The mixture was stirred at room temperature for 4h. The reaction was quenched by the addition of 0.02 g Amberlyst IR-120+ was added and the mixture was filtered through a frit. The amberlyst was washed with additional (3 x 3 mL) MeOH and the combined washings were rotovaped to give 0.138 g of a clear colorless oil. This material was carried on to the next step without further purification. It was redissolved in 8 mL MeOH to which was added 40 mg 10% Pd/C. The mixture was put under an atmosphere of H_2 using a balloon and the reaction was stirred 12 h at rt. After, the solution was filtered through a short pad of celite and washed with additional 2 x 10 mL MeOH. The washings were rotovaped to give 0.131 g of **4** as a clear, colorless oil (100%, two steps). ^{1}H (CD_3OD) δ 400 MHz 4.16 (d, J = 7.6 Hz, 1H), 3.83-3.77 (m, 2H), 3.55-3.49 (m, 1H), 3.47-3.40 (m, 1H), 3.27 (dd, J = 9.0, 9.0 Hz, 2H), 3.18-3.10 (m, 2H), 2.66 (t, J= 7.0 Hz, 2H), 1.65-1.51 (m, 4H); ^{13}C (CD_3OD) δ 100 MHz 105.2, 78.0, 75.0, 71.3, 70.6, 67.0, 42.1, 29.9, 28.1; HRMS









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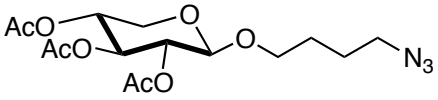

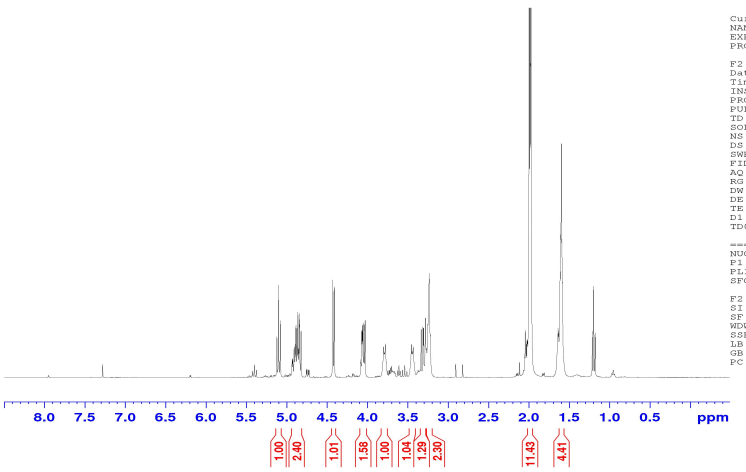


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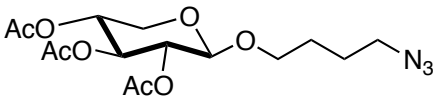

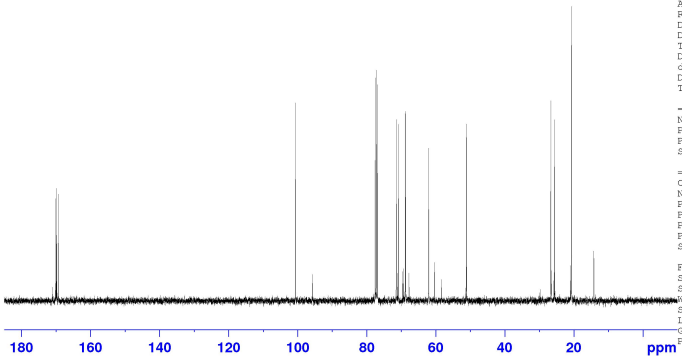


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PL12               19.00 dB
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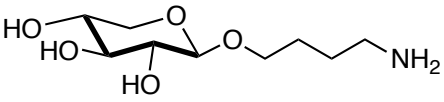

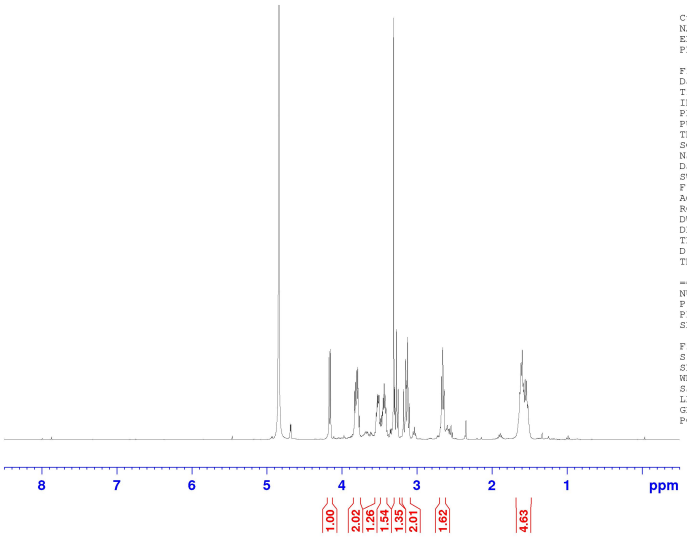


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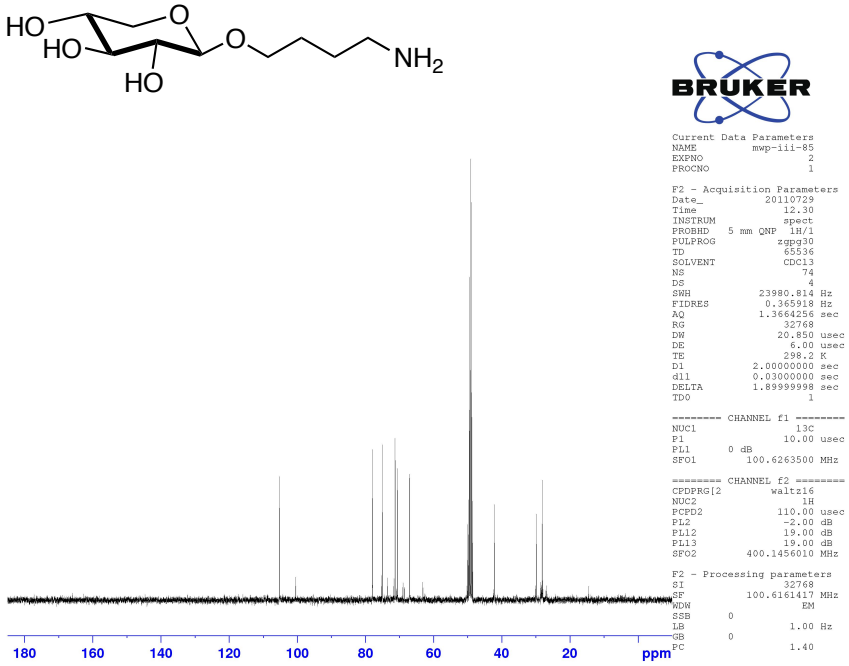

Instrumentation and SPR procedures.

A GWC SPR imager was fitted with a Harvard PHD2000 syringe pump (Item no- 702002) and a Rheodyne chromatographic injection valve (Product No- 9725i, IDEX Health and Sciences LLC) to dispense samples to the array at controlled flow rate. This was the main instrument used in this work for all studies involving IgEs. Binding studies and detection of IgE employed a 250 µL sample loop on the injection valve. Binding events were studied by setting fixed angle of Incident beam according to the method reported elsewhere.[1] Briefly collimated light beam is focused on the prism/gold sensor/flow cell at an angle near the SPR angle. Reflected light is passed through neutral density filter and collected by the CCD camera. Change in reflectivity (SPR response in pixels) upon binding event and real time difference image from the initial mask image was recorded by digital optics V++ software. Difference images were then further processed using NIH imageJ software.

The SpotReady^{TM} sensor array chip features 16 gold spots, which were functionalized as reported previously using 1:9 ratio of monothiol alkane PEG_6-COOH: monothiol alkane PEG_3-OH.[2] The functionalized gold array was then activated by 250 µL 0.4 M EDC + 0.1 M NHS in water on the array for 10-15 mins making sure all gold spots are covered by solution. The array was then rinsed with water and dried in a stream of nitrogen. Then, 1 mg mL^{-1} Ara-h2 peptide, 10 mM β(1,2)-xylose, 1 mg mL^{-1} monoclonal IgE, and 1 mg mL^{-1} bovine serum albumin (control) were immobilized on 4 spots each by placing a 0.5 µL drop of the appropriate solution on the spots and allowing to react for 3 hr in a humidified chamber at ambient temperature. The sensor array was then assembled using a serpentine flow channel to address the spots in the SPRi detector.

A dual channel surface plasmon resonance spectrophotometer, SR7000DC from Reichert Analytical Instruments (NY, USA) was used to study binding kinetics of model chicken IgY on allergens. Allergen epitopes were attached to SPR gold chips with mixed self-assembled monolayer of 90% monothiol alkane PEG_3-OH and 10% monothiol alkane PEG_6-COOH from Reichert

(SR7000 part no- 13206061). Phosphate buffer saline containing 0.05% Tween-20 (PBS-T), pH-7.2 was used as running buffer at 25 ^{o}C. Binding kinetics were measured as described previously.[3]
The carboxyl functionalized gold SPR sensor chip (Reichert SR 7000, part no- 13206061) was activated with freshly prepared 0.4 M 1-ethyl-3-(3-dimethylamino-propyl) carbodiimide (EDC) and 0.1 M N-hydroxysuccinimide (NHS) in water at 20 µL min^{-1}. Subsequently, the Ara h-2 peptide or ethylamine linked β-(1,2) xylose was immobilized onto activated gold surface at 5 µL min^{-1}. After allergen immobilization unreacted excess carboxyl functional groups were masked with 1 M ethanolamine, pH 8.5 at 20 µL min^{-1}. The reference channel was not derivatized with allergens. A 3 s pulse of 0.1 M hydrochloric acid (HCl) at 100 µL min^{-1} was used to remove unbound allergen and ethanolamine. Residual HCl was washed out by flowing buffer for 1 min at 100 µL min^{-1}. Then, 10 mM PBS buffer was injected for time similar to kinetics binding studies between allergen and antibody. This served as a blank with 0 nM antibody concentration and we subtract the same from referenced SPR responses. Anti-peanut antibody from chicken (IgY) was diluted sequentially in PBS buffer to give concentrations ranging from 4nM to 32 nM. IgY solutions were injected into SPR system at 20 µL min^{-1} for an association and dissociation phase of 400 sec each. Antibody and allergen interactions were disrupted using short pulses of 0.1 M HCl solution at 100 µL min^{-1} to regenerate surface for next binding event followed by PBS buffer injection for 2 mins to remove any residual HCL from the system.

Kinetics analysis of epitope binding to IgE antibody. SPRi sensor chip immobilized with peptide, β(1,2) xylose, monoclonal Anti-Human IgE and control BSA was assembled into the SPR imaging system. 10 mM phosphate buffered saline containing 0.05% tween 20 (PBS-T) was used as running buffer. Running buffer was used at 100 µL min^{-1} until a stable base line was achieved. Then 1 M glycine, pH 8.2, at 20 µL min^{-1} flowed through the array for 10 min to quench unreacted EDC derivatives. The array was then pre-conditioned with a 6 sec pulse of 10 mM NaOH at 100 µL min^{-1}, and washed by flowing PBS-T buffer for 2 min. Blank buffer was then injected at 50 µL min^{-1} for times similar to kinetics run and later subtracted from binding response curves. Then, binding of IgE antibodies was monitored at 50 µL min^{-1} for 600 s. After each run, the array surface was regenerated using a 6 s pulse of 10 mM NaOH. Background subtracted response curves were analyzed using Graphpad Prism 5.0 Software.

Synthesis of MP-Ab$_2$ Conjugates. 40 µL of 20 mg mL^{-1} magnetic particles were activated using 1 mL solution of 6 mg mL^{-1} EDC and 2 mg mL^{-1} N-Hydroxysulphosuccinimide in 50 mM pH 5.2 2-(N-morpholino)ethanesulphonic acid (MES) buffer for 10 mins with continuous mixing. Activated carboxyl magnetic particles were washed with 0.01 M PBS buffer 3-4 times and volume was made up to 760 µL. Then 40 µL of 1.2 mg mL^{-1} polyclonal anti-Human IgE antibody was added to react with activated carboxyl groups for 16 hrs. Particles were separated using a Dynamag Spin magnet (invitrogen) and supernatant solution was discarded to remove unbound secondary antibodies. Particles were then incubated for 2 hours with 1000 µL of 5 mg mL^{-1} BSA solution in PBS to block unreacted carboxyl groups to avoid non-specific capture of IgE and serum proteins followed by washings with PBS-T and final volume was made up to 400 µL. Number of detection antibodies per magnetic bead was estimated using Micro BCA^{TM} protein assay kit (Thermo scientific, Product no-23235) as reported previously.[3]

Human IgE capture from serum. Polyclonal Anti-Human IgE antibodies (Ab$_2$) were conjugated by EDC/NHSS to 1 µm magnetic particles (MP) as reported earlier.[4] IgEs from diluted serum were captured on the above MP-Ab$_2$ beads. 40 µL of MP-Ab$_2$ stock dispersion was diluted with 320 µL

of PBS-T buffer (0.05% Tween-20). To this dispersion, 40 µL of human IgE reconstituted in 1000-fold diluted calf serum was added. This mixture was incubated at 37 ^{o}C for 30 min with continuous mixing. To discard unbound IgEs, the mixture was magnetically separated and supernatant fluid was removed. Beads were then washed three times with PBS-T, and final volume was made up to 400 µL with PBS-T before detection.

Detection of IgE using MP-Ab$_2$. A SpotReady 16-spot chip with detection probe and BSA control spots was fitted with a serpentine flow channel. PBS-T was the running buffer. Flow rate of 100 µL min^{-1} was set till stable baseline is achieved followed by injection of 1 M glycine in pH 8.2 buffer at 20 µL min^{-1}. The sensor surface was pre-conditioned by a 6 s pulse of 10 mM NaOH for 5 s at 200 µL min^{-1}. IgE standards captured onto magnetic beads were then injected at 50 µL min^{-1} and the SPR responses monitored. The sensor chip was reused 5-6 times by regenerating the sensor surface after each run with 6 s pulses of 10 mM NaOH at 200 µL min^{-1}. Difference image of baselines after binding event from that of before binding event was recorded to monitor the specificity of binding.

Human serum samples were analyzed using a similar protocol. 10 µL of serum was diluted 1000-5000 times using PBS buffer in order to bring analyte concentrations into the dynamic ranges of the assay calibration graphs. 40 µL of the diluted serum sample was used for protein capture with MP-Ab$_2$. Standardizations for patient samples was done using IgEs reconstituted in similarly diluted calf serum to mimic the effect due to different dilution factor.

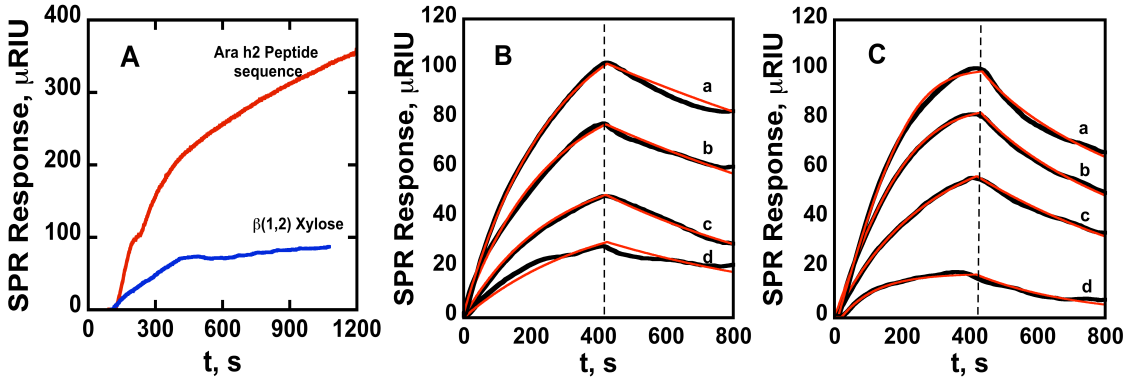

Figure S1. SPR data for binding studies for free IgY at pH 7.2: (A) Responses during covalent attachment for injection of 100 µL of 0.1 mg mL-1 peptide and 0.4 mg mL-1 xylose at 5 µL min-1 on mixed monolayer functionalized sensor surface. (B & C) Association (0-400s) and dissociation (400-800s) curves at 20 µL min-1 (— experimental data, — best fit onto eqs. 1 and 2) showing good fits of the SPR response for a series of antibody concentrations (a- 32 nM, b- 16 nM, c- 8 nM, d- 4 nM) in PBS-T with (B) immobilized Ara h-2 sequence. (C) Immobilized β-xylosyl glycoside.

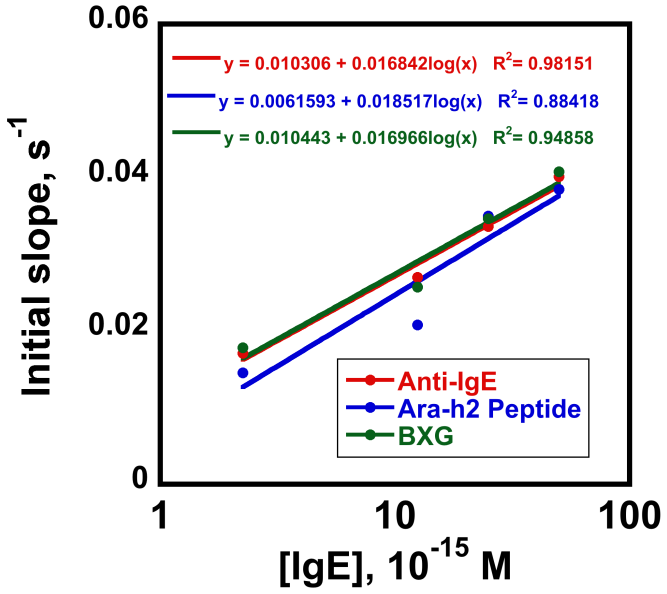

Figure S2. Initial slopes of background-subtracted SPRi response curves vs. concentration of IgEs precaptured on magnetic beads. These data suggest that binding association rates for IgE-Ab_2-MP are very similar on each array spot.

References

[1] T. T. Goodrich, A. W. Wark, R. M. Corn, H. J. Lee. *Methods in Molecular Biology*. Vol. 328. Humana Press: New York, **2006**.
[2] J. Lahiri, L. Isaacs, J. Tien, G. M. Whitesides, *Anal. Chem*. **1999**, *71*, 777-790.
[3] V. Mani, D. P. Wasalathanthri, A. A. Joshi, C. V. Kumar, J. F. Rusling, *Anal. Chem*. **2012**, *84*, 10485–10491.
[4] V. Mani, B. V. Chikkaveeraiah, V. Patel, J. S. Gutkind, J. F. Rusling, *ACS Nano,* **2009**, *3*, 585–594.