A Supramolecular Sensor Array for Selective Immunoglobulin Deficiency Analysis

Yang Liu,[a] Adam D. Gill,[c] Yaokai Duan,[b] Lizeth Perez,[b] Richard J. Hooley[b],[c] and Wenwan Zhong[a],[b]**

[a] Environmental Toxicology Program; [b] Department of Chemistry; [c] Department of Biochemistry and Molecular Biology; University of California-Riverside, Riverside, CA 92521, U.S.A.

richard.hooley@ucr.edu; wenwan.zhong@ucr.edu

Electronic Supplementary Information

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Experimental Section

General Information. Cavitands **1**,[1] **2**,[2] **3**[3] and guest **4**[3] were synthesized according to literature procedures. DSMI **5** (trans-4-[4-(Dimethylamino)styryl]-1-methylpyridinium iodide) was purchased from Sigma-Aldrich (St. Louis, MO) and used without further purification. Immunoglobulin isotypes including IgA, IgG and IgM were purchased from sigma-Aldrich (St. Louis, MO); IgD and IgE were purchased from Abcam (San Francisco, CA). Rabbit anti-human Immunoglobulin (IgA, IgG and IgM) with HRP was also purchased from Abcam. Immunoglobulin depleted human serum was purchased from Celprogen (Torrance, CA). Pierce™ Protein L Magnetic Beads were purchased from Thermo Fisher Scientific (Waltham, MA). All buffers were prepared with ultrapure water.

Principal Component Analysis (PCA) was accomplished with RStudio (Version 1.0.136), an integrated development environment (IDE) for R (version 3.3.2). Jackknife test was also conducted with RStudio. Confidence ellipses were drawn with the data obtained from PCA using Matlab (version R2016b) and a self-developed script.

Experimental Procedures.

Gel electrophoresis. The PAGE gel prepared includes two parts: the top staking gel (4%) and bottom separation gel (13%). 10 μL of immunoglobulin and cavitand mixture was first mixed with 10 μL of 2X Laemmli Sample Buffer (BIO-RAD, Hercules, CA). 12 μL of the above mixture was applied to each lane. The gel was run at room temperature for 30 minutes with 200 voltages. After running, the gel was stained with QC Colloidal Coomassie Stain (BIO-RAD, Hercules, CA), following recommended procedures.

Measurement of Fluorescence Displacement. In a typical displacement assay, 2 μL of the fluorescent guest (**4** or **5**) ([**4**] = 30 μM, [**5**] = 400 μM), 2 μL of the cavitand **1**, **2** or **3** ([**1**] = 40 μM, [**2**] = 50 μM, [**3**] = 50 μM), 4 μL Immunoglobulin protein, and 12 μL of the 1×PBS (10 mM phosphate at pH 7.4 with 150 mM NaCl) were mixed in the 384-well plate, and incubated with mild shaking for 10 minutes. Followed, fluorescence was recorded in a Perkin Elmer Wallac 1420 Victor 2 Microplate Reader (PerkinElmer, Inc., Waltham, MA) with the Ex/Em wavelengths at 530/605 nm for guest **4** or 485/605 for guest **5**.

Tryptophan quenching assay for binding exploration. Tryptophan quenching assay was carried out on a Horiba QM-400 Fluorometer with excitation at 280 nm. With 400 μg/mL Ig

protein in PBS (10 mM, PH 7.4), cavitand **1** concentration was increased from 2.5 μM to 167.5 μM. Emission spectrum was taken after each addition.

Limited proteolysis for binding exploration. Limited proteolysis was used here to study the binding between immunoglobulin and cavitand. This method carries out very brief digestion on the protein to identify which peptides have better accessibility to the protease. Since ligand interaction could either block the peptides at the interface, thus reducing their digestion, or could change protein conformation, releasing more cleavage sites, this method can reveal the peptides locating at the binding interface. Initial inspection of the proteolysis rates of the three Ig proteins, in which the protein was digested by trypsin at various duration (10, 60, 120 min.) and the resulting protein samples were subject to SDS-PAGE, showed that, the presence of cavitand could enhance the digestion efficiency of IgG and IgM, with 20% (for IgG) to 30% (for IgM) being digested even within 10-min proteolysis treatment (see Figure S-3). No obvious change was observed for IgA. Thus, IgM was chosen for limited proteolysis to explore the potential interaction interface. In a typical process, immunoglobulin protein (0.5 mg/mL) was first treated with TCEP and IAA as described above. Then, cavitand (0.5 mg/mL) was added into the treated immunoglobulin protein solution to a final mass ratio of 1:1 and incubated for 1 hour at room temperature. Trypsin was added to above solution with a final trypsin to protein ratio of 1:50. After 10 minutes digestion at 37℃, the reaction was stopped by adding 8 M urea. A final concentration of 5 mM DTT was added and incubated at 60℃ for 30 minutes. After that, IAA was added with a final concentration of 10 mM at incubated for another 20 minutes. The resulted solution was separated with 30 KD Amicon Ultra Centrifugal filter. The passthrough were collected and further digested with trypsin again at 37 ℃ overnight. The sample was cleaned by ziptip before injection into the LC MS/MS (Thermo Scientific LTQ). Data was analyzed by comparing the identified peptide with the protein amino acid sequence.

Protein L bead extraction. The extraction of immunoglobulin from sample solution was carried out following recommended product protocols with small modification. In a typical run, 25 μL beads and 75 μL of TBS buffer (Tris-Buffer saline containing 0.05% Tween-20) were added into 1.5 mL centrifugal tube and vortexed gently to mix. After magnetic pull down, the supernatant was discarded, and another 1 mL TBS buffer was added to do a second wash. After washing, samples with immunoglobulin proteins was added into the pre-cleaned beads and incubated for 1 hour with gentle mixing. The beads were then collected and washed twice with 1 mL TBS buffer.

Elution of bounded immunoglobulin was done with glycine buffer (100 mM, pH 2) and pHs were further neutralized with Tris buffer (1 M, pH 8.5) before applying to the sensor array.

We first tested the effectiveness of coupling Ig protein removal with Protein L beads with Ig protein detection by our array sensor using samples mimicking the deficiencies prepared in 1× PBS buffer. The situation mimicking the normal condition contained all three proteins at the molar ratio of IgA:IgM:IgG = 1:1:8, with the concentrations of IgA and IgM being 200 μg/mL, and that of IgG being 1600 μg/mL. The state of IgA deficiency had IgA:IgM:IgG = 0:1:8; IgM deficiency had IgA:IgM:IgG = 1:0:8; and IgG deficiency contained IgA:IgM:IgG = 1:1:0. The proteins were initially spiked to the 1×PBS buffer, then extracted by Protein L beads. After being eluted off the Protein L beads, they were detected by our sensor array, or ELISA. Once the effectiveness of the method was confirmed, we tested detection of Ig deficiency by spiking the corresponding proteins to serum.

Enzyme-Linked Immunosorbent Assay. ELISA was used here to verify the results of sensor array. In a typical experiment, Ig protein samples were first diluted to a proper concentration (< 20 μg/mL) with PBS buffer. For each well, 50 μL diluted solutions was added and incubate at 4℃ overnight. The solution was removed, and plate was washed twice with PBS buffer before adding the blocking solution (1% BSA in PBS). After 2 hours incubation, BSA was removed and plate was washed twice with PBS buffer. HRP labelled antibody was added and incubate for another 2 hours at room temperature. The plate was washed 4 times before adding the luminescence agents (Pierce™ ECL Western Blotting Substrate, Thermo Fisher Scientific). The chemiluminescence was collected within 10 minutes by the GloMax-Multi Detection System (Promega) with default settings. Standard curve was prepared the same way with series dilution of IgA, IgG and IgM protein stock.

Supporting Figures

1. Discrimination of Immunoglobulin protein in solution.

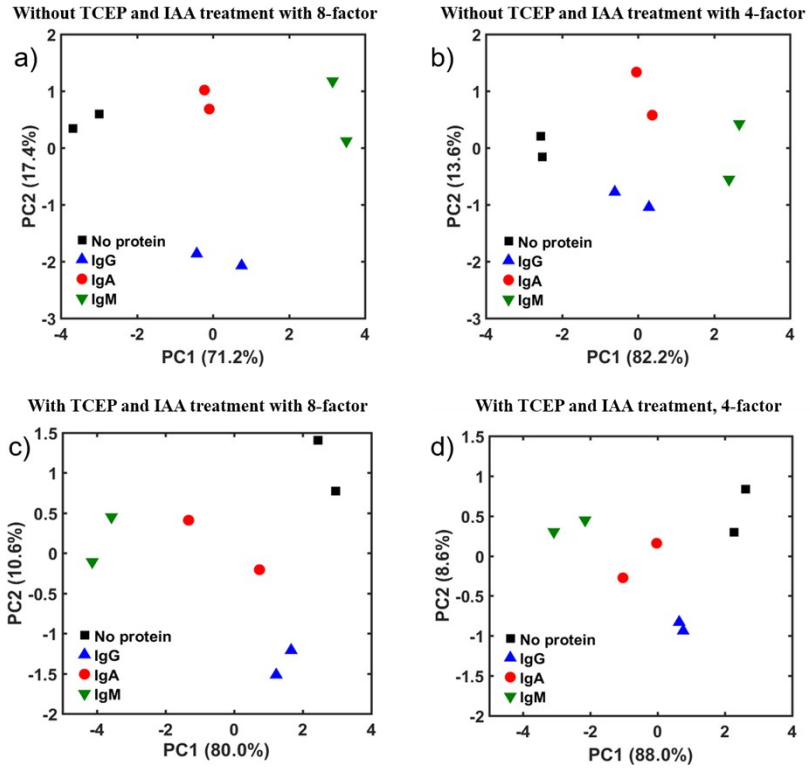


Figure S-1. PCA score plots of data from Figure 2a and 2b with all 8 factors: **1·4**, **2·4** or **3·4** at pH 7.4 or 9.0 and **1·5** at pH 7.4 with [**5**] = 1.5 or 40 μM (a, c) or 4 factors: **1·4**, **2·4** or **3·4** at pH 7.4 and **1·5** at pH 7.4 with [**5**] = 40 μM (b, d).

2. Protein and cavitand binding mechanism study

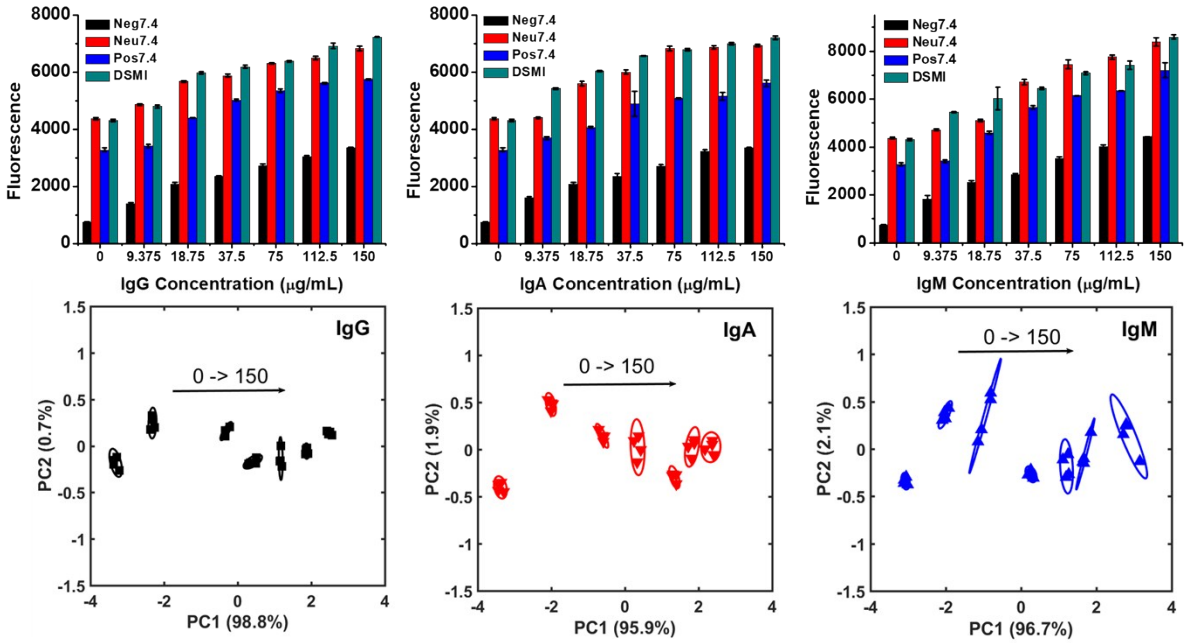


Figure S-2. Raw fluorescence response and PCA plot of IgG, IgA and IgM with 4 factors sensor array. Concentration of proteins are from low to high: 0, 9.375, 18.75, 37.5, 75, 112.5 and 150 μg/mL.

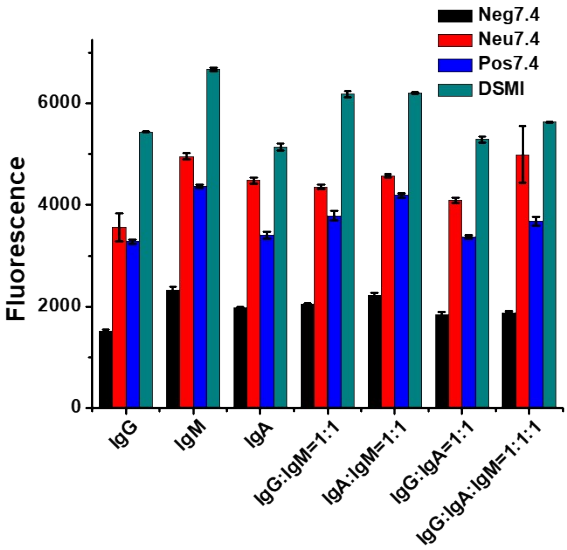


Figure S-3. Raw fluorescence response for protein mixture detection. The total amount of Ig protein in each assay is 150 μg/mL.

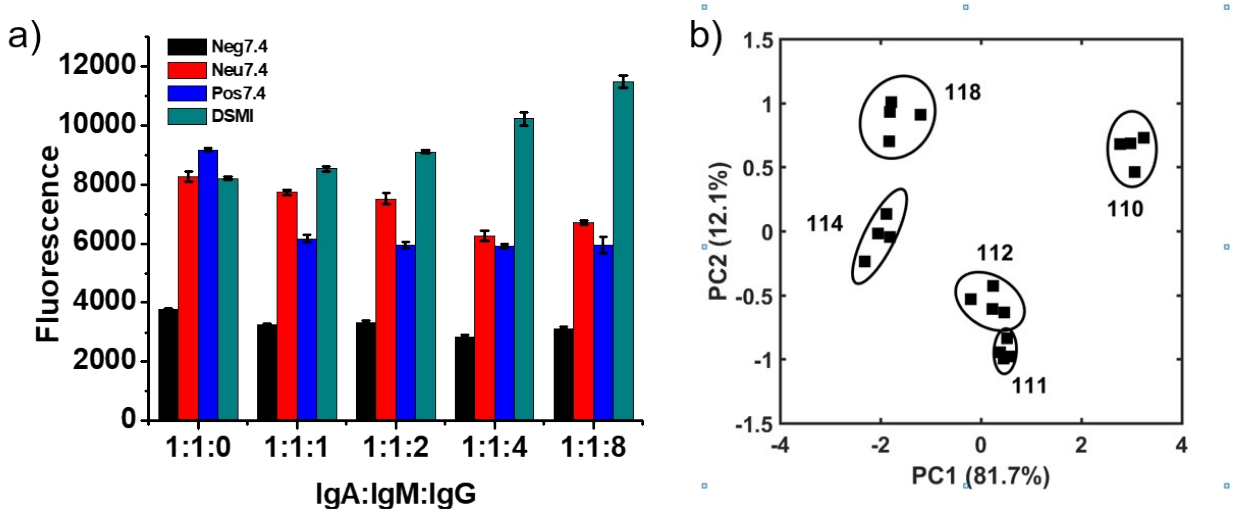


Figure S-4. Detection of IgG in presence of IgA and IgM in 10 mM PBS. IgA = 50 μg/mL; IgM = 50 μg/mL; IgG = 0 – 400 μg/mL.

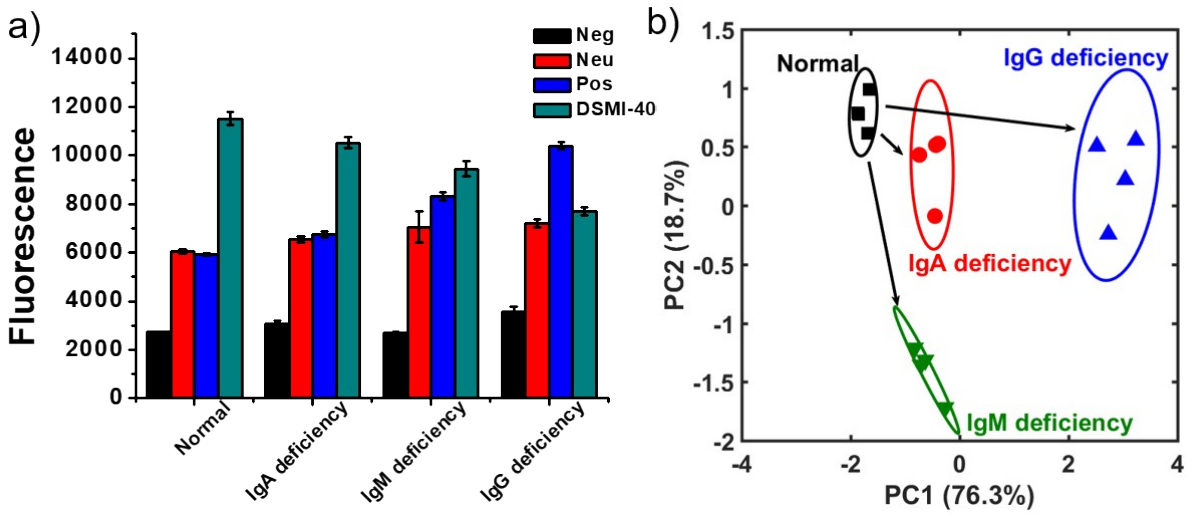


Figure S-5. Mimic of Immunoglobulin deficiencies in 1×PBS buffer. Normal stands for IgA:IgM:IgG = 1:1:8; IgA deficiency stands for IgA:IgM:IgG = 0:1:8; IgM deficiency stands for IgA:IgM:IgG = 1:0:8; IgG deficiency stands for IgA:IgM:IgG = 1:1:0. IgA = 200 μg/mL, IgM = 200 μg/mL and IgG = 1600 μg/mL.

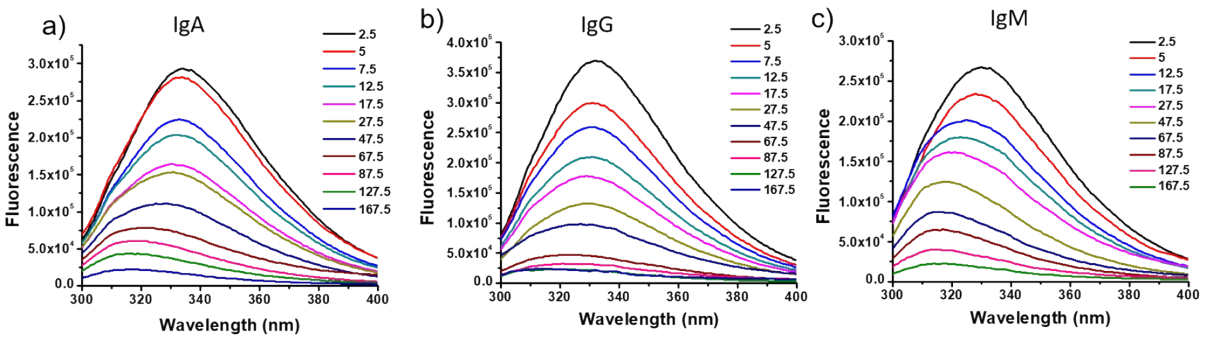


Figure S-6. Cavitand quenching effect of native tryptophan fluorescence of IgA (a), IgG (b) and IgM (c). Excitation = 280 nm. Cavitand concentration increasing from 2.5 µM to 167.5 µM. [protein] = 400 µg/mL

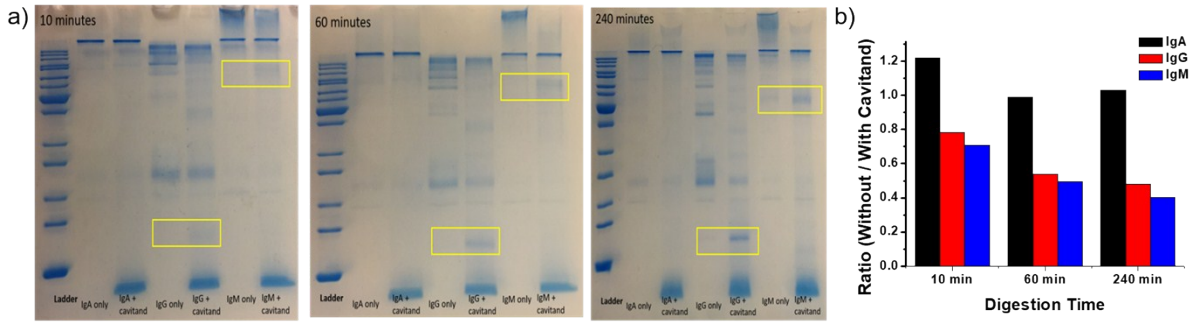
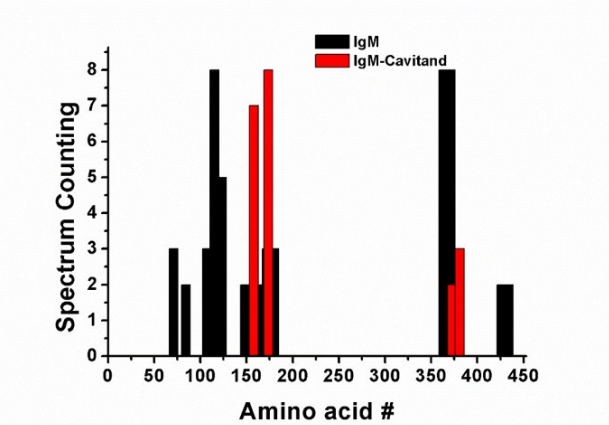


Figure S-7. (a) Trypsin digestion of Immunoglobulin protein with and without cavitand. Protein: cavitand = 1:1 mass ratio. Trypsin: Protein = 1: 50 mass ratios. [Protein] = 200 µg/mL. (b) The bar plot compares the intensity of the product band enclosed in the yellow rectangle.



Amino acid #
1-453

Sequence length	453
Hydrophobicity	80.85
GRAVY	-0.32
MW average	49440.2953 g/mol
MW monoisotopic	49408.5824
Theoretical pI	6.3

Amino acids
Hydrophobic: 39.74%
Acidic: 9.71%
Basic: 11.04%
Neutral: 39.51%

Amino Acid
#75-125

Sequence length	50
Hydrophobicity	43.59
GRAVY	-0.37
MW average	5495.4213 g/mol
MW monoisotopic	5491.8540
Theoretical pI	7.3

Amino acids
Hydrophobic: 48%
Acidic: 12%
Basic: 16%
Neutral: 24%

Amino Acid
#350-400

Sequence length	50
Hydrophobicity	55.52
GRAVY	-0.06
MW average	5536.4513 g/mol
MW monoisotopic	5532.7464
Theoretical pI	7.2

Amino acids
Hydrophobic: 52%
Acidic: 6%
Basic: 8%
Neutral: 34%

Amino Acid #
150-200

Sequence length	50
Hydrophobicity	38.80
GRAVY	-0.60
MW average	5396.0093 g/mol
MW monoisotopic	5392.6081
Theoretical pI	4.5

Amino acids
Hydrophobic: 28%
Acidic: 14%
Basic: 10%
Neutral: 48%

Figure S-8. LC-MS/MS counts of peptides obtained after limited digestion with trypsin. 20 µg/mL protein and cavitand **1** were used.

3. Protein quantification with sensor array

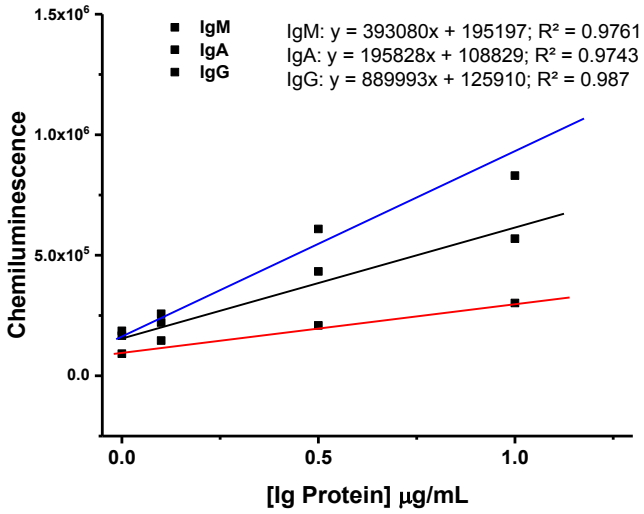


Figure S-9. ELISA calibration curves used for quantification of Ig proteins extracted from serum.

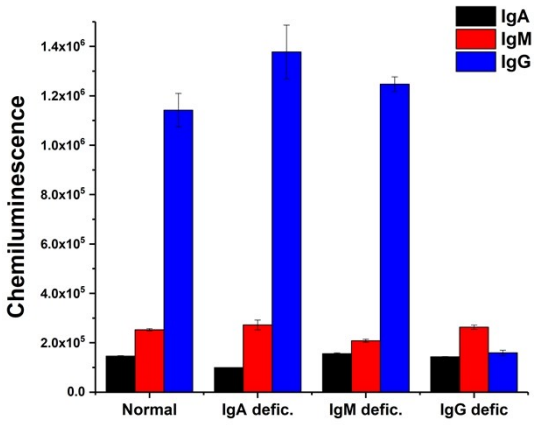


Figure S-10. Chemiluminescence obtained from ELISA for detection of Ig proteins extracted from serum. The protein samples were diluted 400 times before measurement.

Supporting Tables

Table S-1. Fc Region of Five immunoglobulin isotypes. Protein sequences of Fc region were obtained from UniProt. The MW, PI, GRAVY score and Aliphatic Index were calculated using ProtParam tool with default settings.

Protein Fc Region	IgG	IgA	IgM	IgD	IgE
NO. of amino acids	340	346.5	453	384	428
MW	37308	37122	49439	42353	47019
pI	7.8825	5.97	6.35	8.38	8.39
GRAVY	-0.445	-0.237	-0.319	-0.566	-0.375
Aliphatic Index	67.04	72.16	71.39	67.32	67.41

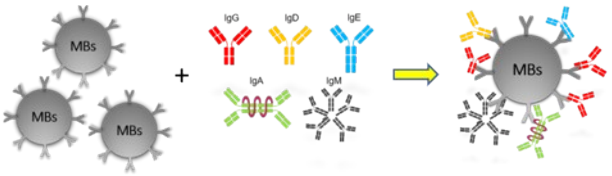
Table S-2. PCA Loading scores for each factor in the 8 factor cavitand guest system.

Factors	**Component 1**	**Component 2**
Neg7.4	-0.376	-0.25
Neg9	-0.382	
Neu7.4	-0.376	-0.179
Neu9	-0.36	0.153
Pos7.4	-0.323	-0.545
Pos9	-0.36	
1.5 μM DSMI	-0.272	0.714
40 μM DSMI	-0.366	0.272

Table S-3. Jackknife analysis of the fluorescence data shown in Figure 2a.

	IgA	**IgD**	**IgE**	**IgG**	**IgM**	**Correctness**
IgA	**4**	**0**	**0**	**0**	**0**	**100%**
IgD	**0**	**4**	**0**	**0**	**0**	**100%**
IgE	**0**	**0**	**4**	**0**	**0**	**100%**
IgG	**0**	**0**	**0**	**4**	**0**	**100%**
IgM	**0**	**0**	**0**	**0**	**4**	**100%**
Total	**4**	**4**	**4**	**4**	**4**	**100%**

Table S-4. Adsorption ratio of protein L beads to each of the three Immunoglobulin protein. 0.125mg beads were used here.

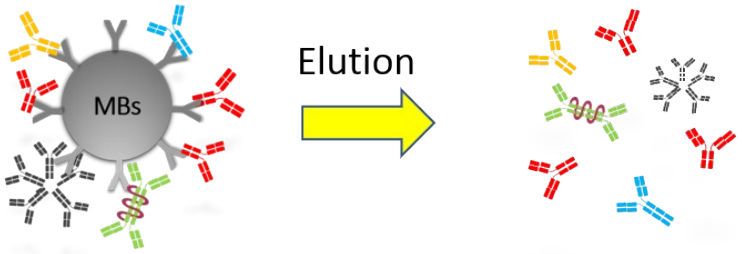


IgG (Total) μg	IgG (adsorbed) μg	Ratio
3.38	1.64	48.73%
6.75	4.23	62.63%
13.50	8.96	66.38%
20.25	13.60	67.15%
31.50	19.40	61.58%
40.50	23.77	58.69%

IgA (Total) μg	IgA (adsorbed) μg	Ratio
3.08	1.02	33.15%
6.16	2.93	47.52%
12.32	6.26	50.79%
18.48	8.09	43.77%
28.75	11.49	39.98%
36.97	13.18	35.64%

IgM (Total) μg	IgM (adsorbed) μg	Ratio
4.67	1.52	32.64%
9.33	3.53	37.81%
20.99	12.38	58.96%
27.99	15.97	57.05%
43.54	23.88	54.84%
55.98	34.83	62.22%

Table S-5. Elution efficiency of IgA, IgG and IgM with protein L beads.



	Total Protein (μg)	Adsorbed (μg)	Eluted (μg)	Elution efficiency
IgA	104.67	63.32	60.98	96.31%
IgG	108.83	71.64	58.66	81.89%
IgM	113.64	59.19	55.69	94.09%

4. References

1. S. M. Biros, E. C. Ullrich, F. Hof, L. Trembleau and J. Rebek, Jr. *J. Am. Chem. Soc.* 2004, **126**, 2870-2876.
2. A. R. Far, A. Shivanyuk and J. Rebek, Jr. *J. Am. Chem. Soc.* 2002, **124**, 2854-2855.
3. Y. Liu, L. Perez, M. Mettry, A. D. Gill, S. R. Byers, C. J. Easley, C. J. Bardeen, W. Zhong and R. J. Hooley, *Chem. Sci.* 2017, **8**, 3960-3970.