A Virus-Enabled Biosensor For Human Serum Albumin
SUPPORTING INFORMATION

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1. Process flow for lithographic preparation of gold electrodes

Figure S1 shows a process flow of lithographically patterned gold films. A detailed description is described in the text.

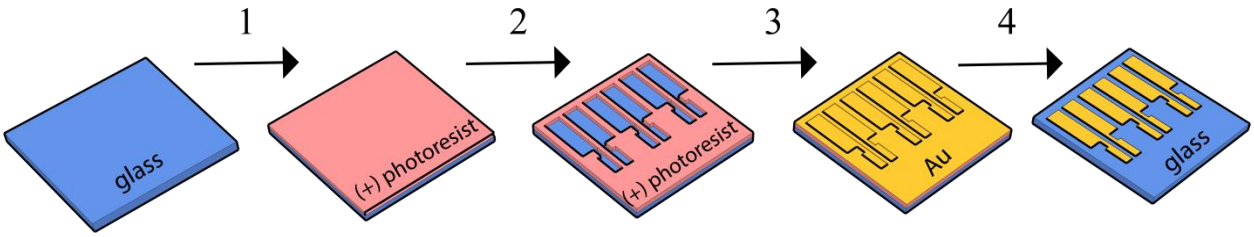

Figure S1. Schematic diagram of gold film electrodes prepared by photolithography. (1) Positive photoresist is spin coated onto a glass substrate, (2) the photoresist is patterned by a photomask and developed, (3) slides are coated with thermally-evaporated gold, (4) and lift off is performed.

2. Enzyme linked immunosorbent assay

Figure S2 shows an enzyme-linked immunosorbent assay for HSA phage binding. Ligand binding is compared to the negative control Stop-4 phage, which shows significantly less binding activity.

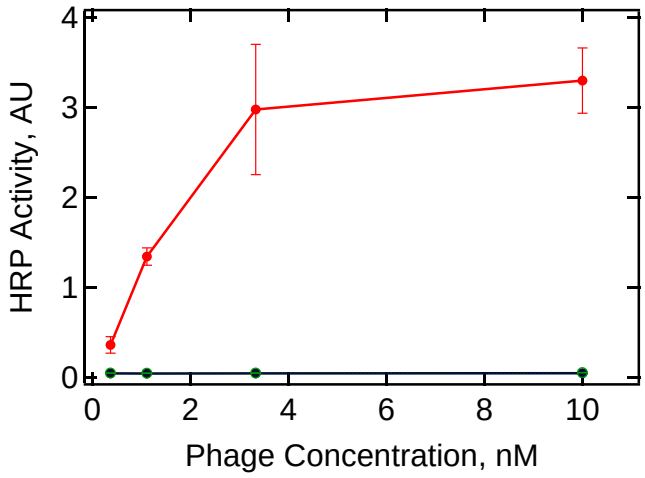

Figure S2. A phage-based ELISA for HSA phage binding. (red) HSA-phage binding to HSA compared to two negative controls, (black) HSA-phage binding to BSA and (dotted green) Stop-4 phage binding to HSA.

3. HSA sensing in buffer

Figure S3 shows the change in the real, $\Delta Z_{re,}$ and imaginary, $\Delta Z_{im,}$ impedance with corresponding signal-to-noise dependence on HSA concentration. The peak *S/N* of 20-140 for this response occurs in the range from 200-500 Hz. Below 100 Hz, ΔZ_{im} shows an even larger increase with increasing HSA concentration – as high as 50 Ω - but noise is also more prominent and the resulting *S/N* is lower than seen for ΔZ_{re}. A more serious problem is that no single frequency provides reliable HSA quantitation across this HSA concentration range using ΔZ_{im}.

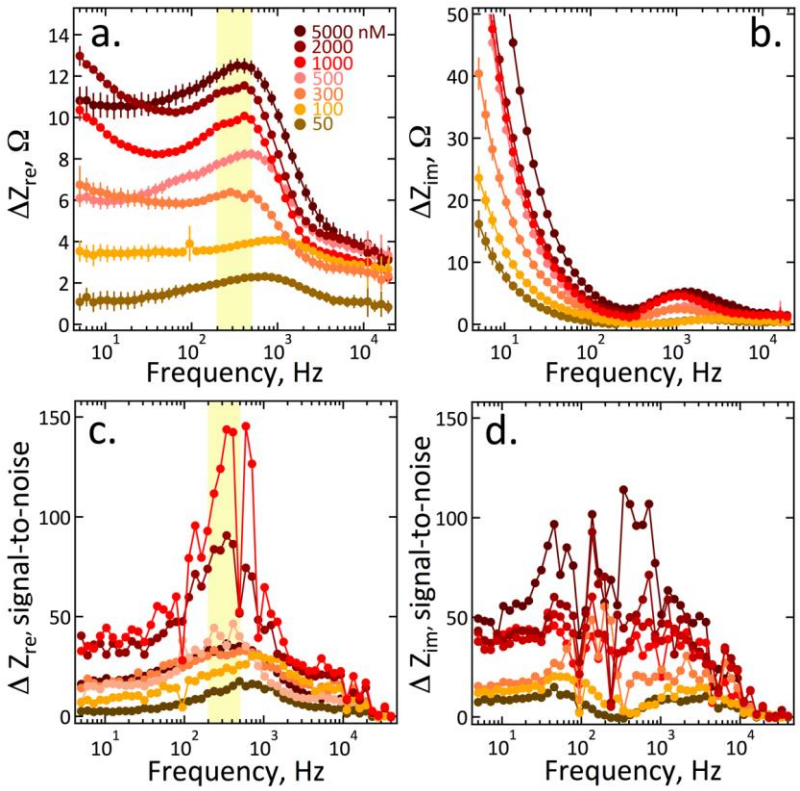

Figure S3. Measurement of ΔZ_{re} affords superior signal-to-noise (S/N) compared with measurement of $\Delta Z_{im.}$ Calibration plots of (a) ΔZ_{re} and (b) ΔZ_{im} versus frequency for virus-PEDOT films in varying concentrations of HSA in run buffer. Each HSA concentration was measured using a different biosensor. Errors bars are defined as the standard deviation, ±1σ, of five consecutive impedance measurements on a single electrode. (c) the relative S/N, defined as $\Delta Z_{re}/\sigma$, *versus* frequency for ΔZ_{re}, and, d) the relative S/N, defined as $\Delta Z_{im}/\sigma$, *versus* frequency for ΔZ_{im}.

4. Effect of pH and blocking agents on non-specific binding

Figure S4 shows a plot of the change in Z_{re} at 340 Hz when exposed to 500 nM BSA under various conditions. Preventing non-specific binding is a critical challenge for non-faradaic impedance based biosensors; in this study pH and blocking agents were explored. Non-specific binding was characterized by exposing a PEDOT film to BSA protein in various buffers. Initial studies of PEDOT films in PBS buffer at pH 8 shows significant non-specific binding to BSA. This response from non-specific binding is reduced as the pH of the buffer is decreased, suggesting that non-specific binding is caused by electrostatic interactions between the positively-charged PEDOT and BSA protein. Non-specific was also reduced by addition of a blocking agent, casein. At pH 8, PEDOT films that were blocked with casein in PBS show less response from BSA than PEDOT films that were not blocked.

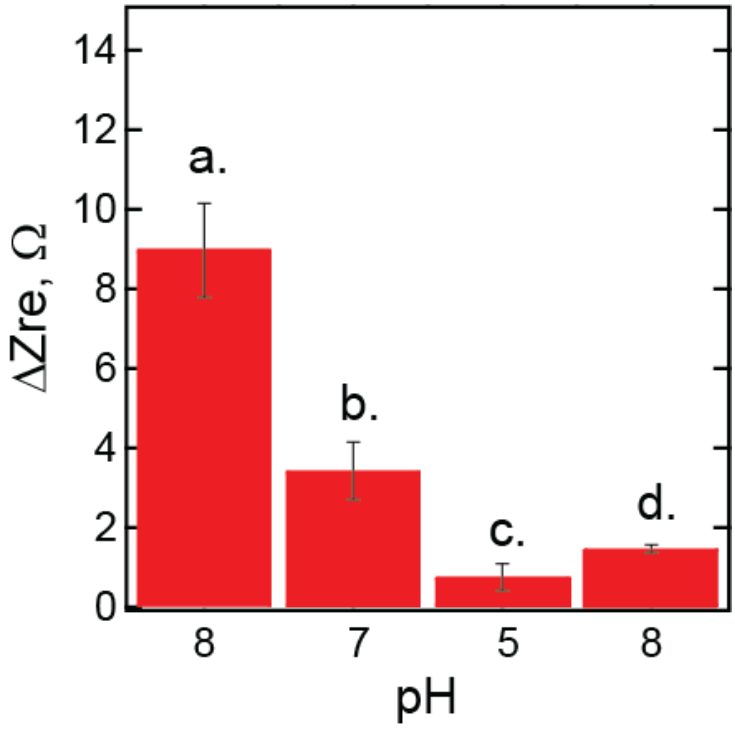

Figure S4. Bar plot of ΔZ_{re} for PEDOT films to determine optimal buffer conditions for reduced non-specific binding. (a-c) PEDOT films, at varying pH's, were equilibrated in PBS and then exposed to 500 nM BSA in PBS. (d) A PEDOT film was blocked with casein in PBS for 15 minutes, equilibrated in PCT, and exposed to 500 nM BSA in PCT at pH 8. Lowering the buffer pH or implementing a casein blocking agent significantly reduced non-specific binding.

5. An equivalent circuit for virus-PEDOT films

Figure S5 shows an equivalent circuit corresponding to virus-PEDOT films on two gold electrodes. R_1, C_2 , R_2, and C_3 represent the two virus-PEDOT films where most of the change induced by HSA binding is in the two resistors. There is little change in the R_2, the solution resistance, and C_1, the geometrical capacitance between the two films. A constant phase element, Q, represents deviation from a perfect capacitor caused by surface roughness or composition differences between the phage and PEDOT the films.

Table S1. Values for elements in the equivalent circuit shown in Fig S1a.

Solution	**C_1 ($x10^{-9}$ F)**	**R_1 (Ω)**	**C_2 ($x10^{-6}$ F)**	**R_2 (Ω)**	**C_3 ($x10^{-5}$ F)**	**Q_1 ($x10^{-4}$ F)**	**n_1**	**R_3 (Ω)**	**Q_2 ($x10^{-5}$ F)**	**n_2**
PCT	2.0 ± 0.4	90 ± 12	7.4 ± 0.4	276 ± 3	3.1 ± 0.5	9 ± 15	0.8 ± .2	92 ± 13	1.41 ± .04	0.710 ± .006
500 nM HSA	2.4 ± 0.6	95 ± 9	6.2 ± 0.5	278 ± 3	3.2 ± 0.8	9 ± 9	0.8 ± 0.4	100 ± 11	1.37 ± 0.05	0.993 ± .008
Change	+ 0.5	+ 5	- 1.2	+ 2	+ 0.1	- 2	0.0	+ 8	- 0.04	+ 1.53

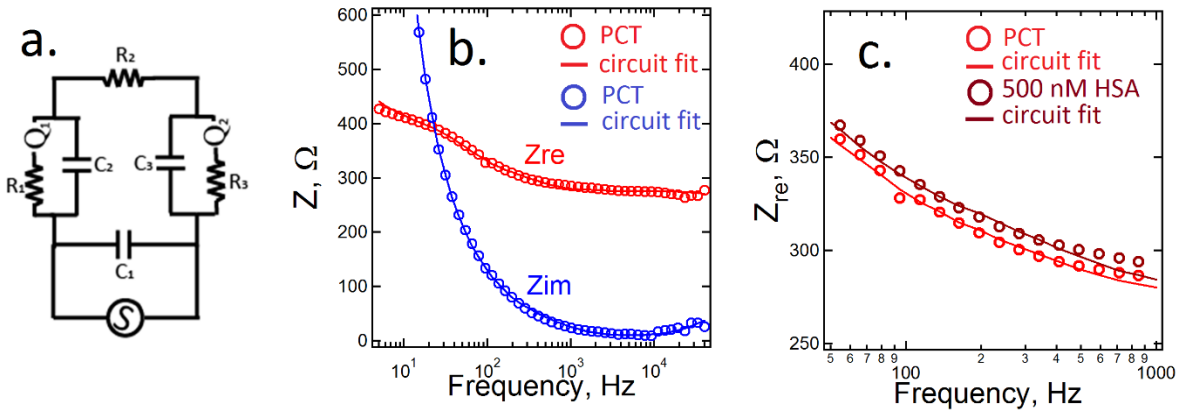

Figure S5. (a) Diagram of an equivalent circuit used to model virus-PEDOT films on two planar-gold electrodes. Circuit elements represent: capacitance between the two electrodes (C_1), the solution resistance of PBS buffer (R_2), the impedance imposed by one virus-PEDOT film (R_1,C_2, Q_1), and the impedance imposed by the second virus-PEDOT film (R_3, C_3, Q_2). (b) Plot of impedance versus frequency for Z_{re} (red) and Z_{im} (blue). The simulated impedance (solid line) data produced by the parameters in Table S1 is plotted on top of the raw impedance data (open circle) of a virus-PEDOT film in PCT buffer. (c) Plot of Z_{re} versus frequency for the range of frequencies,50 Hz to 10 kHz, where a response to HSA binding is observed. Both the raw and simulated data show an increase in impedance from virus-PEDOT films in PCT buffer (red) to 500 nM HSA in PCT buffer (dark red).

6. HSA sensing in synthetic urine

Figure S6 includes analysis of signal-to-noise and R^2 values for the Hill equation for virus-PEDOT films in synthetic urine. ΔZ_{re} increases monotonically with HSA concentration from 100 nM to 5 µM in synthetic urine. Peak ΔZ_{re} response, signal-to-nose, and $R^2 > 0.95$ are observed at 136 Hz.

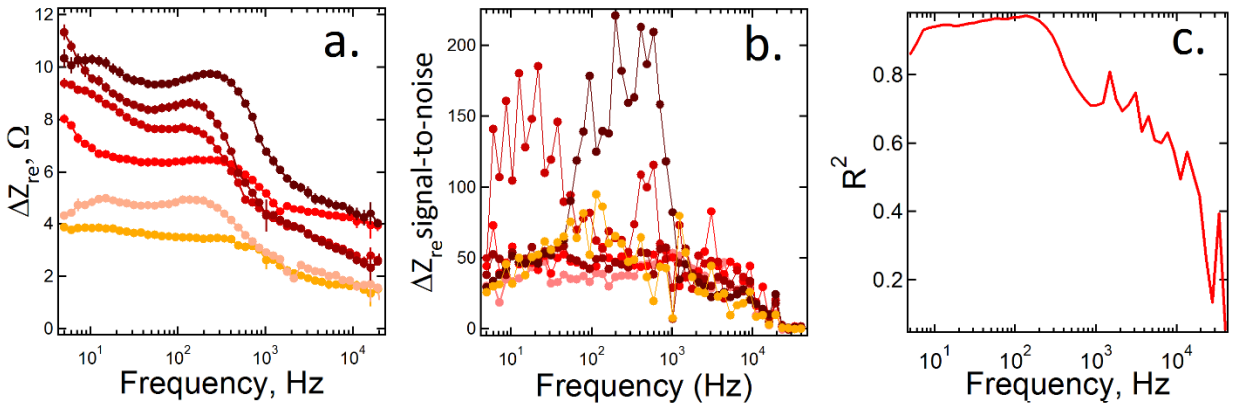

Figure S6.. (a) Calibration plot of ΔZ_{re} versus frequency for virus-PEDOT films in varying concentrations of HSA in synthetic urine. Each HSA concentration was measured using a different biosensor. Errors bars are defined as the standard deviation, ±1σ, of five consecutive impedance measurements on a single electrode. (b) S/N, defined as $\Delta Z_{re}/\sigma$, versus frequency for ΔZ_{re}. (c) At each frequency, ΔZ_{re} versus [HSA] was fitted to the Hill equation and the square of the regression coefficient, R^2, versus frequency plot. $R^2 = 1$ represents the best fit of the Hill equation to the data.

Materials and Methods

Materials. All chemicals and solvents were purchased from Sigma Aldrich and used as received, unless noted. Nichromix solutions (Godax Laboratories) were prepared in sulfuric acid (Macron Fine Chemicals) by package directions. Positive photoresist (Shipley S-1827) and developer MF-219 (Microchem Corporation), gold pellets (5 N purity, Kurt J. Lesker Co.), and chromium powder (3 N purity, American Elements) were used for the photolithography of gold films. Devices were O_2 plasma cleaned using a basic plasma cleaner (PDC-32G, Harrick Plasma). Flow cells were designed and manufactured by Wainamics Inc., Fremont CA. Milli-Q UV water (ρ >18 MΩ cm) was used as the solvent for all aqueous solutions. Phosphate-buffered saline (PBS) buffer (137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4 pH 8) was filtered through a 0.22 µM pore size membrane (Corning). The wash buffer was 0.1% Tween 20 (Fisher Scientific) in PBS. 2 mg/mL of Casein in PBS was used as blocking solution. Human serum albumin (HSA) of purity >97% based on SDS-PAGE was used as received. Bovine serum albumin (BSA, Calbiochem Omnipur) was used as received. The buffer for all blank and analyte solutions used for EIS measurement contained 2 mg/mL casein and 0.1% Tween 20 (henceforth "tween") in PBS buffer. Synthetic urine (Ricca Chemical Co.) solutions composed of 18.01 g/mol water, 60.05 g/mol urea, 58.44 g/mol sodium chloride, magnesium sulfate heptahydrate, and 147.02 g/mol calcium chloride dihydrate.

Device Fabrication. Gold-film electrodes on glass substrate were fabricated by photolithography (Figure S1). 1 in. by 1 in. glass slides were soaked in nichromix solution overnight, rinsed with Millie Q-UV water, and dried with pure air. Each slide was spincoated with positive photoresist and baked in a 90 °C oven for 30 min. The slides were then patterned using a contact photomask, 365 nm UV light source, and alignment stage (Newport, 83210i-line, 4 s), developed (MF-319), and rinsed with Millie Q-UV water. A 2 nm thick layer of chromium followed by a 60 nm thick layer of gold were thermally evaporated onto the slides. The slides were then soaked in acetone and sonicated for 10 min to lift off the photoresist layer and subsequently rinsed with Millie Q-UV water. Each 1 in by 1 in slide contained three pairs of gold electrodes, and were cut into three separate devices.

AFM and SEM Analysis. Scanning electron microscopy (SEM) was performed on uncoated films using a FEI Magellan 400L XHR SEM operating at 2 keV. Atomic force microscopy (AFM) images of PEDOT-only and phage-PEDOT films were acquired using an Asylum MFP-3D-SA atomic force microscopy (Asylum Research, Santa Barbara, CA) equipped with Olympus AC160TSAFM tips (Olympus) in laboratory ambient air. AC Mode AFM images were obtained over a 20 µm range at 512 x 512 pixels. Images and amplitude traces were analyzed using the Asylum image processing software.

Phage Library Design and Selection of HSA Binders.

Phage Library Design. The HSA binding phage was selected from a mega random peptide library (MRPL) created by pooling 24, individually constructed, peptide libraries (Table S2). Individual peptide libraries contain five to 18 amino acids codons encoding the 20 naturally occurring amino acids or none or two cysteines. Each library has a theoretical peptide diversity of 10^6 to 10^{23}, respectively. Among the library members, X_8 is the only designed linear library, and the other libraries are structurally constrained by disulfide bonds. The library of peptides is fused to the N-terminus of P8, which is localized in the oxidizing environment of the periplasm prior to assembly in the phage[1, 2].

Table S2. The MRPL components representing theoretical and actual peptide diversity.

Library Design	Number of Residues	Theoretical Peptide Diversity	Actual Peptide Diversity
CX_5C	7	3.2×10^6	1.7×10^8
CX_5CX	8	6.4×10^7	1.2×10^8
$X_2CX_2CX_2$	8	6.4×10^7	2.0×10^8
X_8	8	2.6×10^{10}	2.3×10^8
XCX_5C	8	6.4×10^7	1.8×10^8
CX_5CX_2	9	1.3×10^9	1.0×10^8
$X_2CX_2CX_3$	9	1.3×10^9	2.0×10^8
$X_2CX_3CX_2$	9	1.3×10^9	3.2×10^8
X_2CX_5C	9	1.3×10^9	4.7×10^8
$X_2CX_4CX_2$	10	2.6×10^{10}	2.5×10^8
$X_2CX_5CX_2$	11	5.1×10^{11}	3.2×10^8
$X_2CX_6CX_2$	12	1.0×10^{13}	2.7×10^8
$X_2CX_7CX_2$	13	2.0×10^{14}	3.3×10^8
$X_2CX_8CX_2$	14	4.1×10^{15}	2.5×10^8
$X_2CX_9CX_2$	15	8.2×10^{16}	3.3×10^8
$X_2CX_{10}CX_2$	16	1.6×10^{18}	3.8×10^8
$X_4CX_2GPX_4CX_4$	18	1.6×10^{18}	1.2×10^8

$X_4CX_{10}CX_4$	20	2.6×10^{23}	6.0×10^{7}
$X_5CX_8CX_5$	20	2.6×10^{23}	1.2×10^{8}
$X_5CX_9CX_4$	20	2.6×10^{23}	1.0×10^{8}
$X_6CX_6CX_6$	20	2.6×10^{23}	1.2×10^{8}
$X_6CX_7CX_5$	20	2.6×10^{23}	2.5×10^{8}
$X_7CX_4CX_7$	20	2.6×10^{23}	1.2×10^{8}
$X_7CX_5CX_6$	20	2.6×10^{23}	1.1×10^{8}

A highly efficient site-directed mutagenesis method, Kunkel mutagenesis was used to clone libraries in the phagemid.[3] The pooled libraries were electroporated, into SS320 cells, a strain of *E. coli* optimized for efficient electroporation.[4] The P8 phagemid, carries an antibiotic resistance marker, bacterial and phage origins of replication, and a phage packaging signal. Virions extruded from co-infected cells contain single-stranded recombinant phagemid DNA, and display a mixture of recombinant and wild-type P8 proteins.[5] Phage-displayed peptides have a physical linkage between phenotype (the expressed peptide) and its encoding phagemid DNA. This linkage provides easy access to the DNA sequence of any MRPL phage-displayed peptide that binds a target of interest.

The cells were grown in 2YT media supplemented with carbenicilin (50 μg/ml) and tetracyclin (5 μg/ml), and infected with KO7 helper phage (10^{10} phage/mL) before growth in 2YT/carbenicilin media supplemented with kanamycin (20 μg/ml). The culture was shaken at 250 rpm for 16-18 h at 37 °C. To isolate the phage from the cells, the culture was centrifuged for 10 min at 10 krpm at 4 °C. The supernatant was decanted into separate tubes, and the phage was precipitated by addition of 1/5th volume of PEG-NaCl (2.5 M NaCl, 20% PEG-8000). The solution was placed on ice for 1 h. Next, the phage was recovered by centrifugation for 10 min at 10 krpm. The phage pellet was resuspended in phosphate-buffered saline (PBS, 140 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO4, 2 mM KH_2PO_4, pH 7.2) with addition of 0.05% Tween-20. After additional centrifugation for 10 min at 15 krpm, the phage precipitation step was repeated as described above. Phage were isolated by PEG-NaCl precipitation, and their concentration was determined by UV absorbance at 268 nm.

Selection of HSA Binders

In each of five rounds of selection, 15 wells of a 96-well microtiter plate (Nunc) were coated with 10 μg/mL HSA in 100 μL of PBS (pH 8.0), and incubated overnight at 4 °C on a shaker. After removal of the coating solution, 400 μL of a solution of 0.2% w/v casein in PBS was used to block the wells for 30 min on a shaker at room temperature. In successive rounds, the blocking reagent was switched to BSA, ovalbumin, or nonfat milk (NFM). The plate was then washed three times with 300 μL per well 0.05% Tween-20 in PBS. Phage were added to the wells in a buffer containing 0.2% w/v BSA, 0.05% Tween-20 in PBS. After 90 min incubation on a shaker at room temperature, the wells were washed with 0.05% Tween-20 in PBS. The numbers of washes increased with each round from 3, 5, 5, 7, and 9 times respectively for the five rounds. The bound phage was eluted by adding 100 μL of 0.1 M HCl and shaking

vigorously at room temperature for 5 min. The phage were neutralized with 33 μL of 1 M Tris-HCl, pH 8.0. Before incubation for 45 min at 37 °C, 2 mL of the eluted phage was used to infect 20 mL of log phase *E. coli* XL-1 Blue cells. Helper phage KO7 was added at $\approx 6X10^{12}$ phage/mL, and after 45 min of incubation, the culture was transferred to 200 mL of 2YT supplemented with 50 μg/mL carbenicillin and 20 μg/mL kanamycin and shaken overnight at 37 °C for 16-18 h.

After 5 rounds of selection, spot assays were performed on around 200 selectants targeting HSA (10 μg/mL). The assay was performed based on a sandwich ELISA format in 96-well microtiter plates. Four potential HSA binders were obtained from the selections and spot assay. The four peptides were further examined for specificity to HSA. In the specificity assay, the selectants were screened against seven different proteins including kinases, membrane proteins, and high pI proteins. Furthermore, to increase accuracy, the binders were also screened against *E. coli* and mammalian cancer (fibroblasts, kidney, and prostate) cell lysates. From the selected variants, only two binders demonstrated high affinity and specificity to the HSA protein.

The apparent K_d of both binders was calculated by dose–dependent ELISA. The phagemid containing the genes encoding P8 fused to either peptide 1 or 2 were transformed in $CaCl_2$ competent *E. coli* XL-1 Blue cells. Similar steps were followed for the phage growth and precipitation as mentioned in the previous section. The phage concentration was determined by UV absorbance at 268 nm. For the ELISA, 96-well microtiter plates (Nunc) were coated with 10 μg/mL of HSA in PBS (pH 8.0), and incubated on a shaker at 4 °C overnight. The wells were blocked with 0.2% w/v solution of BSA in PBS at room temperature on a shaker for 30 min and washed three times with wash buffer (0.05% v/v Tween-20 in PBS). The phage were then serially diluted along with a negative control (Stop4 phage) in phage dilution buffer (0.2% w/v BSA, 0.05% v/v Tween-20 in PBS). The plates were incubated with the samples at room temperature on a shaker for 1 h and then washed five times with wash buffer. Anti-M13/HRP conjugate (GE Healthcare) was diluted 1:5000 in the phage dilution buffer, added to the wells, and incubated for 30 min on a shaker at room temperature. The wells were washed four times with wash buffer and once with PBS. 100 μL of a solution of 2 mg/mL o-phenylenediamine dihydrochloride, 0.02% w/v H_2O_2, in citric acid buffer (50 mM citric acid, 50 mM Na_2HPO_4, pH 5.0) was added to each well. After 10 min incubation, the absorbance at 450 nm was measured using a microtiter plate reader (Bio-Tek). The data was further analyzed by Prism (GraphPad) software, which estimated the apparent K_D for the binders. Since, the apparent K_D of binder 2 (0.08 nM) was higher than binder 1 (45 nM), future experiments were conducted with binder 2. For incorporation of phage in the virus-PEDOT films, the phage pellet obtained after the above protocol was re-suspended in aqueous $LiClO_4$ (12 mM) solution.

The mega random peptide library (MRPL) from which HAS binders were selected was constructed from 24 individual peptide libraries. Each library was designed to be structured, yet as unbiased as possible, and thus provide potential binders for a wide range different targets. The individual libraries were designed to be structurally different using degenerate codons and varied placement of cysteine-based disulfide bonds. Each amino acid residue in the peptide was randomly assigned using NNS as the codon designation; where N is any nucleotide, and S is C or G. Thus, each degenerate position is designed to encode all 20 natural amino acids, termed "hard randomization." The codon choice prevents the occurrence of the non-suppressible stop codons TGA and TAA that could result in non-displayed peptides. Due to the degeneracy of the genetic code, some amino acids will be represented twice (A, G, P, T and V; in one-letter amino acid code) or three times (R, L and S). In addition, a TAG stop codon in each position could be

encoded. In an amber suppressor strain of *E. coli* such as XL1 Blue used here, the TAG will be read as mixture of glutamine and a stop codon minimizing the impact of terminating translation to <50%.

The constrained libraries have random residues flanked by cysteines, which spontaneously form disulfide bonds creating constrained loops of two to ten amino acids. In addition, the libraries are constructed to limit the number of possible loop conformations to improve the overall free energy of binding compared with the unconstrained library. The design can increase binding affinity by limiting the entropic cost upon peptide binding to the target. Also, the X_8 linear library is included to provide conformations missing from the constrained libraries. Therefore, engineering and using multiple primary libraries with unconstrained and constrained peptides forming large or small loops, MRPL has the diversity to yield productive results when applied to selections against a variety of targets. The theoretical diversity of the MRPL far exceeds the capability of any known system to accommodate full expression and maintenance of 10^{24} individual library members.

The twenty-four libraries were mixed and subjected to thermodynamic or equilibrium-based selections for binding to HSA. The target protein, human serum albumin (HSA) was purchased from Sigma Aldrich as lyophilized powder, and was dissolved in PBS (pH 8.0) for rounds of selection. After bio panning targeted to HSA, a total of 200 selectants were screened from the different rounds of selections. Furthermore, the binders were tested for specificity to HSA against various proteins aurora kinase A (AKA), bovine serum albumin (BSA), non-fat milk, ovalbumin, hen egg white lysozyme (HEWL), and cav(1-104)[6] by phage-based ELISA. To increase stringency for specificity, cell lysates of *E. coli* and human cancerous cell lines (LnCAP, 3T3, 293T, and PC3) were also included in the specificity assay.

Table S3. Sequence of the binders selected after affinity maturation and specificity assay.

Binder	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**	**14**	**15**	**16**	**17**	**18**	**19**	**20**
1	Q	Y	G	E	A	C	W	D	G	Y	S	W	K	N	C	L	A	L	T	L
2	D	C	P	I	Y	C	E	D	G	Y	C	L	R	K	C	V	D	L	Y	R

Two binders of 20 residues each were selected with high affinity and specificity to HSA (Table S3). Binder 1 and 2 emerged from round 5, with binder 2 being cysteine rich. Binder 2 had four cysteines, compared to binder 1, which had two cysteines (Table S3). This probably suggest that intramolecular disulfide bond formation within the peptide might have advantage in binding HSA to acquire binding energy. The apparent K_D of both the binders through phage-based ELISA was calculated in sub-nanomolar range. Binder 2, which exhibited the strongest relative affinity and specificity to HSA in the ELISA, was chosen for further bio-sensing studies.

References

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