

1 Designing Fluorescent Peptide Sensors with Dual Specificity for the 2 Detection of HIV-1 Protease

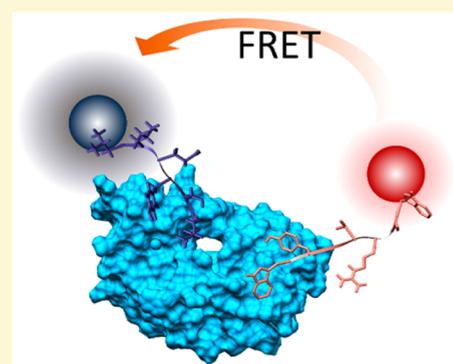
3 Karla-Luise Herpoldt,[†] Arbel Artzy-Schnirman,[†] Andrew J. Christofferson,[‡] Adam J. Makarucha,[‡]
4 Roberto de la Rica,^{†,§} Irene Yarovsky,^{*,‡} and Molly M. Stevens^{*,†}

5 [†]Department of Materials, Department of Bioengineering, Institute of Biomedical Engineering, Imperial College London, Prince
6 Consort Road, London SW7 2AZ, United Kingdom

7 [‡]Health Innovations Research Institute, RMIT University, GPO Box 2476, Melbourne, Victoria 3001, Australia

8 **S** Supporting Information

9 **ABSTRACT:** HIV-1 protease is a key enzyme in the life cycle of HIV/AIDS, as it
10 is responsible for the formation of the mature virus particle. We demonstrate here
11 that phage-display peptides raised against this enzyme can be used as peptide
12 sensors for the detection of HIV-1 protease in a simple, one-pot assay. The
13 presence of the enzyme is detected through an energy transfer between two
14 peptide sensors when simultaneously complexed with the target protein. The
15 multivalent nature of this assay increases the specificity of the detection by
16 requiring all molecules to be interacting in order for there to be a FRET signal.
17 We also perform molecular dynamics simulations to explore the interaction
18 between the protease and the peptides in order to guide the design of these
19 peptide sensors and to understand the mechanisms which cause these
20 simultaneous binding events. This approach aims to facilitate the development
21 of new assays for enzymes that are not dependent on the cleavage of a substrate
22 and do not require multiple washing steps.



23 ■ INTRODUCTION

24 Human immunodeficiency virus type 1 (HIV-1) is the most
25 common cause of acquired immune deficiency syndrome
26 (AIDS) worldwide, with some 2.1 million new cases being
27 diagnosed in 2013.¹ HIV-1 protease (HIV-1 PR) is a dimeric
28 enzyme from the family of aspartic proteases. It is a crucial
29 enzyme in the lifecycle of the HIV virion, being responsible for
30 the cleavage of the precursor polyproteins into the mature virus
31 particle.² The virus remains ineffective without the presence of
32 an active form of this enzyme, leading it to be considered as the
33 major clinical target for antiretroviral therapies.³ Although it has
34 been widely exploited as a drug target and exhibits broad
35 substrate recognition, only a few studies document the use of
36 the enzyme as a biomarker for HIV infection.^{4–7} While these
37 methods report detection limits in the low picomolar range
38 (LOD < 1 pM), they are limited in their effectiveness as point-
39 of-care diagnostics given their reliance on detection mecha-
40 nisms involving complex instrumentation such as surface
41 plasmon resonance (SPR) or quartz crystal microbalance
42 (QCM). Furthermore, in these approaches the signal is
43 generated by the cleavage of a peptide substrate by the HIV-
44 1 PR, and therefore, they are also susceptible to false positives,
45 since the peptides can be cleaved nonspecifically by other
46 proteases.

47 Traditional HIV diagnostics are divided into three main
48 categories: those which detect the patient's antibodies against
49 HIV, those which detect the p24 antigen, and those which rely
50 on RT-PCR for the detection of viral load DNA.⁸ While these

techniques are highly sensitive, they too suffer from the 51
requirements of expensive equipment and reagents as well as 52
highly skilled, laboratory-trained staff, making it unsuitable for 53
use in resource-limited settings. A full discussion of HIV 54
diagnostic tools is beyond the scope of this Article, but 55
comprehensive reviews can be found by Suaifan et al.⁹ and 56
Cornett and Kirn.¹⁰ 57

Förster resonance energy transfer (FRET) has long been 58
used as a tool to study active enzymes, in particular, proteases.¹¹ 59
We have previously reported the use of FRET to detect the 60
presence of disease related enzymes in serum.^{12,13} In these 61
studies the single event that is monitored is the cleavage of a 62
peptide tagged with a pair of fluorescent FRET probes. The 63
peptide acts as the substrate for the enzyme of interest so that 64
in the presence of the enzyme the peptide is cleaved and the 65
FRET disappears. FRET is well-suited for this detection as 66
energy transfer will only occur between two nonradiatively 67
coupled fluorophores if they are within 10 nm of each other, 68
allowing detection from a single recognition event to occur. 69
However, this same advantage can limit the complexity of 70
systems that can be studied since the detection probe must be 71
carefully designed to enable an efficient signal. Due to the 72
strong distance dependence on energy transfer efficiency (r^6), it 73
can be challenging to design an enzyme substrate that is short 74

Received: September 17, 2015

Revised: September 24, 2015

75 enough to fall within this range but long enough to be resistant
76 to nonspecific cleavage.
77 Herein we describe a FRET-based method of detection of
78 HIV-1 protease using multiple recognition elements which does
79 not rely on peptide cleavage (Figure 1). These peptide sensors

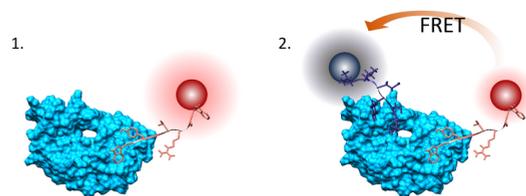


Figure 1. Schematic of the detection mechanism of HIV-1 protease. (1) A fluorescent nanosensor binds specifically to the HIV-1 PR molecule (peptide nanosensor 1 WSRVGYW-AF647). (2) A second nanosensor (peptide nanosensor 2 LLEYSL-BHQ-3), tagged with a fluorescent quencher, interacts with the active site of the protease. The biorecognition events trigger an energy transfer process between peptide nanosensors 1 and 2, and the signal from the fluorescent dye decreases.

80 are designed to interact independently but in close proximity of
81 each other, allowing the generation of a strong FRET signal.
82 The use of multiple biorecognition events also increases the
83 specificity of the assay since FRET will only occur when
84 multiple sensors are bound to the same HIV-1 PR molecule.

85 Our sensors rely on peptide biorecognition elements: short
86 peptide sequences that recognize HIV-1 PR. These peptide
87 sequences are then labeled with fluorescent dye molecules to
88 actuate the detection signal. The position of the interaction
89 between the first peptide sensor and the protease is determined
90 by choosing a known inhibitory peptide sequence of the
91 enzyme (LLEYSL, identified by Lee and Maruyama).¹⁴ Being a
92 competitive inhibitor peptide, it must interact in the active site
93 of the enzyme. A selection of complementary recognition
94 peptides that interact with other regions of the protein was
95 identified through phage display. Phage display is a powerful
96 technique to identify peptide sequences which show an affinity
97 for a particular target. While it has been most successful in
98 identifying sequences which bind inorganic surfaces,¹⁵ it has
99 also been heavily utilized to identify sequences that can have
100 applications in drug discovery¹⁶ and toward the design of
101 synthetic antibody libraries.¹⁷ Phage-display derived peptides
102 have also successfully been used as biorecognition elements in
103 biosensors.¹⁸

104 While peptides have an intrinsically lower binding affinity
105 than larger protein molecules such as antibodies¹⁵ on account
106 of their lack of secondary structure, they remain well-suited to
107 their use as protein recognition elements due to their simple
108 chemical structure and ease of synthesis compared to large
109 proteins. They can also be easily incorporated into more
110 complex nanomaterials¹⁹ as they are more stable to harsh
111 environments such as low pH or temperature which can alter
112 the conformation of protein-based materials. This makes them
113 ideal for use in sensing applications, especially with regard to
114 point-of-care diagnostic tools in resource-limited settings.

115 ■ MATERIALS AND METHODS

116 **Experimental Methods.** *Enzymes.* Recombinant HIV-1 protease
117 was purchased from BioVendor and used as received. Lyophilized, salt-
118 free pepsin (Sigma-Aldrich) at >2500 units/mg was resuspended in
119 the appropriate assay buffer at 4 mg/mL. Lyophilized Papain

(Worthington Biochemicals) was prepared to 20 units/mL in the
appropriate assay buffer.

Phage Display. A Ph.D.-7 phage display kit (New England Biolabs)
was purchased, and the recommended protocol²⁰ was followed. In
brief, 2.4 μ M HIV-1 protease was coated onto the surface of a well of
Nunc-Immuno MicroWell 96 well plate in 0.1 M NaHCO₃ and left at
4 °C overnight. The well was then washed and blocked with a blocking
buffer of 5 mg/mL bovine serum albumin (BSA) in TBS for an hour.
After washing to remove bound proteins, 5 μ L of the phage library was
added to each well in 95 μ L Tris buffered saline with 0.05% (v/v)
Tween-20, pH 7.4 (TBST). The phage was allowed to interact for an
hour before nonbound phage was removed through vigorous washing.
Bound phage was eluted with 0.2 M glycine-HCl (pH 2.2) including 1
mg/mL BSA. The eluted phage was then neutralized with the addition
of 1 M Tris-HCl (pH 9.1) and then added to 20 mL of *E. coli* ER2738
and left to grow for 5 h for amplification. The eluted phage was
quantified by plating these *E. coli* on LB XGal/IPTG plates. Since the
library phages are derived from the M13mp19 vector, which carries the
lacZa gene, phage-infected plaques acquired a blue color when plated
on media containing Xgal (5-bromo-4-chloro-3-indoyl-*b*-D-galacto-
side) and IPTG (isopropyl-*b*-D-thiogalactoside). Colored plaques were
picked, and DNA was sequenced from these plates (sequencing was
carried out by GATC Biotech, Germany). Subsequent biopanning
rounds were then carried out by applying the eluted and amplified
phage to plated HIV-1 protease. Three rounds of biopanning were
carried out in this manner.

Peptides. Sensor peptides were synthesized by standard automated
Fmoc solid-phase peptide synthesis as described earlier. Synthesis used
a Rink-amide solid resin on a PTI Quartet peptide synthesizer. The
peptides were cleaved and deprotected with 95:2.5:2.5 trifluoroacetic
acid (TFA)/triisopropylsilane/H₂O for 3 h and precipitated and
washed with cold diethyl ether. The crude peptides were purified to
>95% (determined by LC-MS) on a preparative C₁₈ HPLC column
using a water/acetonitrile mobile phase containing 1% (v/v) TFA.
Biotinylated peptides were purchased at >95% purity from Genscript.

ELISA. Nunc-Immuno MicroWell 96-well solid plates were coated
with 1.2 μ M HIV-1 protease in 0.1 M NaHCO₃ and incubated
overnight at 4 °C. BSA was prepared at 1 mg/mL and coated in the
same buffer as a control. Wells were then blocked with 2% (w/v) skim
milk for 1 h at room temperature and washed well with PBST (0.05%
(v/v) Tween-20). Biotinylated peptides, dissolved in PBS with 10%
(v/v) DMSO, were then added at 100 μ g/mL to each well and left to
incubate for 1 h at room temperature. The plate was then washed
again in PBST before the addition of streptavidin-linked hydrogen
peroxidase and left to incubate for 45 min. The presence of the peptide
was quantified by the addition of a tetramethylbenzidine (TMB)
colorimetric substrate, and the absorbance at 450 nm was measured.

Inhibition Assay. An assay to measure the inhibition of HIV-1
protease was performed using a fluorogenic substrate of HIV-1
protease,²¹ purchased from Sigma-Aldrich. This is a synthetic peptide
sequence that contains the HIV-1 PR cleavage site (Tyr-Pro) and two
covalently modified amino acids labeled with a FRET pair (EDANS, 5-
2(aminoethylamino)-1-naphthalenesulfonate and DABCYL, 4,4'-dime-
thylaminoazobenzene-4-carboxylate). The substrate was prepared in a
500 μ M stock solution in dimethyl sulfoxide (DMSO). Assays were
performed at room temperature in a buffered solution of 0.1 M sodium
acetate, 1.0 M sodium chloride, 1.0 mM ethylenediaminetetraacetic
acid (EDTA), 1.0 mM dithiothreitol (DTT), 10% (v/v) DMSO, and 1
mg/mL BSA at pH 4.7. Enzyme and substrate concentrations were
kept constant at 240 nM and 10 μ M, respectively. Measurements of
the intensity at 490 nm were taken over 3 min in a FluoroLog
spectrophotometer (Horiba).

Fluorophore-Peptide Labeling. Alexa Fluor 647 was purchased as a
succinimidyl ester from Life Technologies. The dyes were dissolved to
1 mg/mL in dimethyl sulfoxide and coupled at a 1:1 ratio with purified
peptides including 1 equiv of 1-ethyl-3-(3-(dimethylamino)propyl)
carbodiimide (EDC) and 0.2 equiv of 4-dimethylaminopyridine
(DMAP). The reaction was left overnight at room temperature. The
solution was then diluted 10 \times into an acidic mobile phase of water/
acetonitrile containing 0.1% (v/v) TFA and purified on a semi-

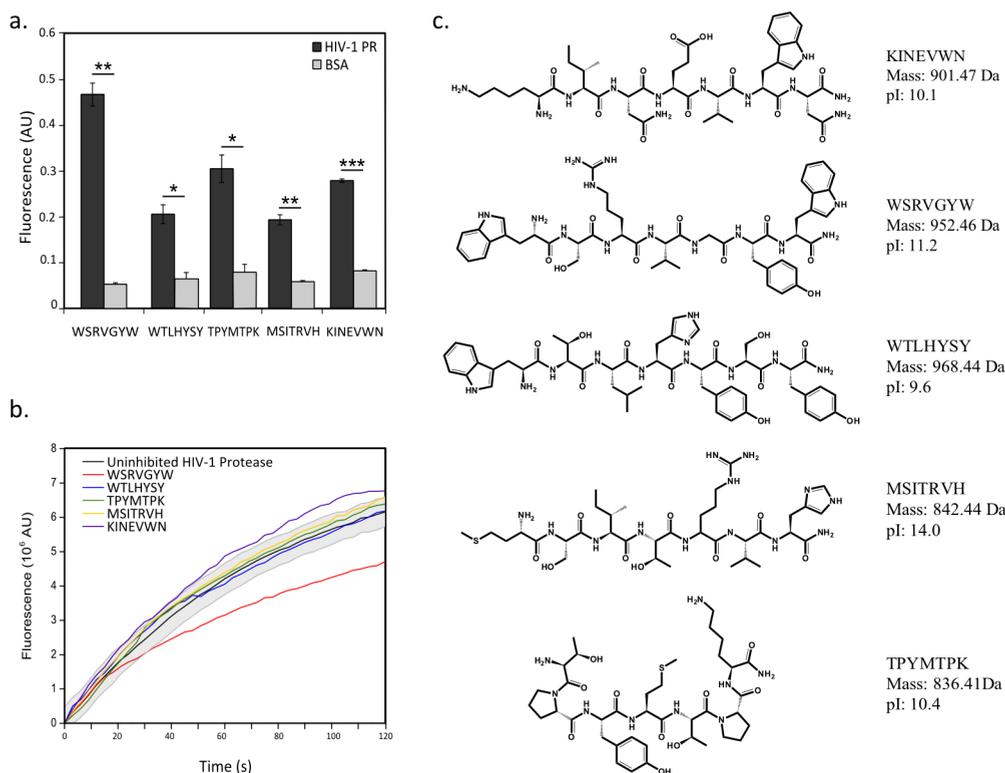


Figure 2. Characterization of five peptide sequences isolated from phage display as potential binders to HIV-1 protease. (a) ELISA showing binding of sequences to HIV-1 protease compared to BSA as a control protein. Error bars shown are standard deviation ($n = 3$). Significance is given by a two-tailed student t test. * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.005$. (b) Competitive inhibition assay of sequences in the presence of a large excess of phage-display peptides ($10 \mu\text{M}$). The peptide WSRVGYW partially inhibits the enzyme at high concentrations. (c) Amino acid structures of selected peptides.

190 preparative HPLC using a C_{18} column. Colored fractions were
 191 collected and analyzed by UV-vis spectroscopy to identify the single
 192 fraction containing peaks at 220 and 280 nm (indicative of the peptide
 193 backbone) and 647 nm (corresponding to the Alexa Fluor dye).
 194 Peptides conjugated to these dyes were not seen through LC-MS or
 195 MALDI as has been reported previously,²² and a full discussion can be
 196 found in the Supporting Information (Figure S9). BHQ-3 as a
 197 succinimidyl ester was purchased from Biosearch Technologies and
 198 conjugated in the manner described above. Fractions collected after
 199 HPLC purification were identified via LC-MS showing an MW of
 200 1443.7 (expected 1410.59, observed mass in positive mode = $M +$
 201 methanol + H^+).

202 NHS-fluorescein and NHS-rhodamine were purchased from Pierce
 203 Biotechnology. The dyes were dissolved in DMSO to 100 mM. The
 204 dyes were then added at a 1:1 ratio to the protected peptides on resin.
 205 The dyes were allowed to couple overnight at room temperature in
 206 dimethylformamide (DMF) with $25 \mu\text{L}$ of N,N -diisopropylethylamine
 207 (DIPEA). The peptides were then cleaved from the resin and purified
 208 as described above. Fractions collected after HPLC were identified via
 209 LC-MS showing an MW of 1093.5 (LLE-fluor, expected: 1094.27,
 210 observed mass in negative mode = $M - H^+$) and 683 (WSR-rhod,
 211 expected: 1364.6, observed mass in positive mode = $M + 2H^+$).

212 **FRET Surface Assay.** Amine functionalized glass slides (Sigma-
 213 Aldrich) were immersed in 0.1 M sodium bicarbonate (pH 8.6) with
 214 10% (v/v) glutaraldehyde and left for 1 h. The slides were then
 215 washed thoroughly with deionized water before being dried with
 216 nitrogen. HIV-1 protease was prepared at $50 \mu\text{g}/\text{mL}$ in 0.1 M sodium
 217 bicarbonate with 10 mM sodium cyanoborohydride. A $10 \mu\text{L}$ portion
 218 of the protein solution was spotted onto the prepared glass slide
 219 and left for 3 h in a humidity chamber. The slides were then washed
 220 thoroughly with deionized water. Unreacted amines were blocked with
 221 a solution of 1 M ethanolamine in 0.1 M sodium bicarbonate with 1%
 222 (w/v) BSA (at pH 8.6). The slides were left to block for 1 h and then
 223 washed thoroughly. The slide was immersed in WSR-647 for 1 h in the

dark before being washed in PBST (0.05% (v/v) Tween-20), before 224
 225 being imaged at 700 nm with a Li-Cor Odyssey infrared scanner. Half
 226 the slide was then immersed in LLE-BHQ-3 while the other half was
 227 left in PBST for an hour in the dark. The slide was then washed
 228 thoroughly with PBST again and reimaged. The intensity of the spots
 229 was quantified using Li-Cor Image Studio.

In Solution FRET. LLE-fluor and WSR-rhod at $20 \mu\text{M}$ were added 230
 231 to varying concentrations of HIV-1 PR in the PR assay buffer
 232 described above, adjusted to pH 6 to a final volume of $60 \mu\text{L}$ in the
 233 wells of a 384 clear-bottom black microwell plate. The plate was
 234 incubated in the dark at 4°C for an hour before the fluorescent spectra
 235 were read on a SpectraMax M5 plate reader (Molecular Devices). The
 236 solution was excited at 480 nm, and the emission was read between
 237 510 and 600 nm with a cutoff value of 495 nm.

Computational Methods. Structure Preparation. For the HIV-1 238
 239 protease, crystal structures with the protease flaps open (PDB ID
 240 2PC0), semiopen (PDB ID 1HHP), and closed (PDB ID 1HVC)
 241 were used. Two side chains were changed (I3V and C95A) in 1HHP
 242 to match the native protease sequence. For 1HVC, the structural,
 243 active-site water molecule, HOH-415, was retained for docking, but all
 244 other crystallographic water was removed. Hydrogens were added to
 245 the structures using AutoDockTools (ADT) from Autodock 4.2.²³

Docking. Initial configurations for the peptide-protease complexes 246
 247 were generated using the docking program Autodock 4.2, with default
 248 settings used unless otherwise noted. Three overlapping grids were
 249 defined on the basis of areas of interest (Supporting Information,
 250 Figure S6). WSRVGYW and LLEYSL were used as ligands, with
 251 Gasteiger charges assigned by ADT. Each peptide was docked into
 252 each grid for the three protease structures, with a maximum of
 253 10 000 000 energy evaluations and 27 000 genetic algorithm
 254 generations using the Lamarckian Genetic Algorithm. For each
 255 docking run, the top 100 structures based on the Autodock estimation
 256 of free energy of binding were examined, and the 3D structures of the
 top 10 were evaluated for geometric fit variation to select the 257

258 distinctive starting structures for subsequent molecular dynamics
259 (MD) simulations.

260 **Molecular Dynamics.** MD simulations were performed using the
261 Amber 12 MD code²⁴ and Amber force field.²⁵ The systems were
262 solvated with a TIP3P water²⁶ box extending at least 12 Å from the
263 complex resulting in a box containing approximately 34 000–36 000
264 atoms. Chloride ions were added to neutralize the overall charge.
265 Asp25 in the catalytic site was protonated to mimic the PR in its native
266 configuration.²⁷ Bond lengths involving hydrogen were constrained
267 using the SHAKE algorithm.²⁸ The particle-mesh Ewald scheme was
268 used for the electrostatic interactions, with a nonbond interaction
269 cutoff (electrostatics and van der Waals) of 8 Å.

270 Prior to dynamics, the systems were minimized using the steepest
271 descent algorithm, followed by a conjugate gradient method, with a
272 convergence criterion of 10^{-4} kcal/mol Å. Position restraints of 2 kcal/
273 mol Å² were maintained on the protein and peptide atoms during
274 minimization and equilibration. Equilibration consisted of 50 ps of
275 constant-volume MD as the system was heated from 0 to 300 K,
276 followed by 50 ps of constant pressure MD. Unrestrained simulations
277 of up to 20 ns were performed for each system, with atomic
278 coordinates saved every 10 ps. A 2 fs time step was used. Temperature
279 was maintained at 300 K by a Langevin thermostat, and pressure was
280 maintained at 1 atm by a Berendsen barostat.

281 ■ RESULTS AND DISCUSSION

282 To identify peptide sensor 1, phage display was carried out
283 against immobilized HIV-1 PR. A Ph.D.-7 phage-display library
284 from New England Biolabs containing approximately 10^{11}
285 clones of 10^9 individual sequences was incubated against the
286 target protein. Three rounds of biopanning were carried out,
287 and enrichment was observed (Supporting Information, Figure
288 S1). Some 32 clones were then selected for sequencing and
289 were then assessed for binding to HIV-1 PR. Binding was
290 established through an enzyme-linked immunosorbent assay
291 (ELISA) against the M13 bacteriophage, and from this, five
292 candidates were selected and sequenced. These were then
293 synthesized and further characterized (Figure 2). Through an
294 ELISA-like enzyme linked binding assay utilizing biotinylated
295 peptides, all five candidates showed preferential binding to
296 HIV-1 PR compared to an immobilized control protein (bovine
297 serum albumin, BSA). A competitive inhibition assay was then
298 used to determine whether any of the peptides altered the
299 proteolytic activity of HIV-1 PR, in order to determine whether
300 they interacted near to the active site of the peptide, where
301 peptide sensor 2 is located (Figure 1). The FRET signal
302 between peptide sensors 1 and 2 is distance dependent, and
303 therefore peptides interacting in the vicinity of the active site
304 may result in the highest FRET efficiency. One of the
305 sequences (WSRVGYW) shows mild inhibitory effects with a
306 K_i of 229 μM (Supporting Information, Figure S2).

307 Given the single active site of the protease, the competitive
308 inhibition shown in Figure 2b implies that WSRVGYW must
309 interact in the vicinity of the active site. It was thus identified as
310 the most promising sequence found through phage display
311 since it is expected to be positioned close to peptide sensor 2,
312 which should maximize the FRET efficiency. Peptide sensor 1
313 was fabricated by synthesizing WSRVGYW and conjugating a
314 fluorescent molecule, Alexa Fluor 647 (AF647), to the N-
315 terminus. Peptide sensor 2 was fabricated by synthesizing the
316 inhibitory sequence LLEYSL, to a Black Hole Quencher-3
317 (BHQ-3), also through the N-terminus. Both sensors form a
318 FRET pair in which BHQ-3 acts as a dark quencher for the
319 fluorescent AF647 dye. Thus, if both peptide sensors are
320 simultaneously interacting with a single protease molecule, a
321 decrease in the fluorescence of AF647 should be observed.

HIV-1 PR was immobilized on amine functionalized glass 322
slides using glutaraldehyde coupling. After unreacted groups 323
were blocked, the slide was incubated for an hour with peptide 324
sensor 1 at a concentration of 210 μM . After thoroughly 325
washing the membrane for an hour to remove nonspecifically 326
bound dye, the slide was imaged (Figure 3a). The interaction 327

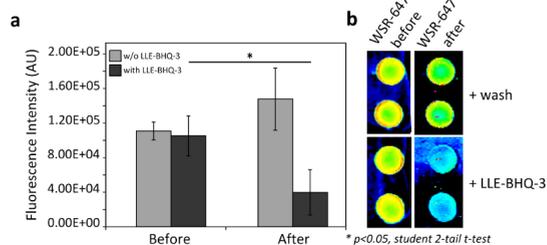


Figure 3. On surface FRET showing detection of HIV-1 PR using two independent recognition events. (a) Quantification of fluorescence quenching on surface. Error bars shown are standard deviation ($n = 3$). Significance is given by a two-tailed student t test. (b) The presence of immobilized HIV-1 PR can be seen through the fluorescence signal of nanosensor 1 (WSR-AF647). On addition of nanosensor 2 (LLE-BHQ-3) the fluorescence signal is quenched. No such signal decrease is observed through continued washing.

between the peptide sensors 1 and the HIV PR can be clearly 328
observed in Figure 3a. When the slide was incubated with 329
peptide sensor 2 at a concentration of 609 μM , the fluorescent 330
signal was quenched (Figure 3b). The decrease in fluorescence 331
was not seen when the peptide was incubated with a nonlabeled 332
version of LLEYSL, which demonstrates that it is originated by 333
FRET and not by competition for the biorecognition of the 334
protease (see Supporting Information, Figure S3). This 335
supports the hypothesis that both peptides are binding to the 336
protease giving multiple independent recognition events. 337
Furthermore, when the membrane was incubated in the 338
working buffer, no signal decrease was observed, which 339
demonstrates that the decrease in fluorescent signal did not 340
originate by washing peptide sensor 1 off the protein (Figure 341
3b). No FRET was observed when the two peptide sensors 342
were incubated in the absence of HIV-1 PR (Supporting 343
Information, Figure S3). These experiments demonstrate that it 344
is possible to detect HIV PR with the proposed detection 345
scheme based in the dual biorecognition by peptide sensors. 346

The peptide sensors can detect immobilized HIV-1 PR at the 347
concentration of 800 nM or higher (Supporting Information, 348
Figure S4). This concentration value is likely dictated by the 349
 K_{off} of the WSR peptide ($K_d = 44.6 \mu\text{M}$). The binding affinity 350
of the LLEYSL peptide is much stronger ($K_d = 3.04 \text{ nM}$), and 351
hence, the sensitivity of the assay will lie between these two 352
values (Supporting Information, Figure S5). Peptides found 353
through phage display are well-known for displaying low to 354
moderate binding affinity.¹⁵ 355

We next performed molecular dynamics (MD) simulations in 356
order to ascertain whether it would be possible for both 357
peptides to interact with the enzyme's active site simulta- 358
neously, giving rise to the observed FRET signal and to confirm 359
our design principles. 360

Initial molecular docking of the inhibitor peptide LLEYSL 361
and the best binding sequence from phage display to the 362
protease was performed using Autodock 4.2²³ in order to 363
identify energetically favorable starting structures of the 364
molecular complexes which were then simulated using classical 365

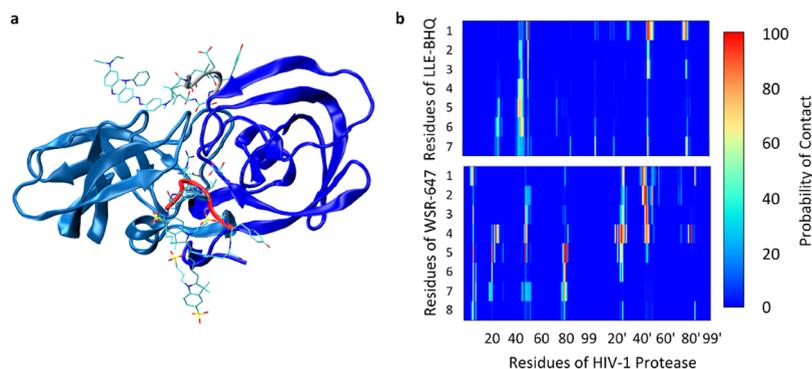


Figure 4. Simulated structure of HIV-1 PR complexed with peptide nanosensors: (a) The most populated cluster from REST simulations showing both peptide–dye conjugates bound to the protease flaps on opposite sides of the active site. WSRVGYW is shown in red, and LLEYSL is shown in green. (b) Contact maps showing the interaction probabilities with HIV-1 PR for each residue of the nanosensors for the most populated cluster of the peptide–dye conjugates. Residue 1 represents the contacts between the dye or quencher to the protease, and the peptides are subsequently numbered from N-terminus to C-terminus.

366 all-atom molecular dynamics using Amber 12.²⁴ Three
367 conformations of the protease were used, namely, with its
368 flaps open, semiopen, and closed. For each conformation, three
369 areas were electrostatically mapped to explore interaction areas
370 for the peptides: the active site, the hinge region, and the
371 “bottom” of the enzyme (Supporting Information, Figure S6).

372 For each peptide/flap/grid combination the top 100 docking
373 orientations were ranked using an estimated free energy of
374 binding, and for each combination the 3D structures of the top
375 10 best binders were examined. As a result, a total of 15
376 protein–ligand complexes were chosen for molecular dynamics
377 simulation, on the basis of the docking score, specificity of the
378 docking poses, and ligand contacts with the protease. Some
379 peptide/flap/grid combinations did not yield any complexes
380 with favorable free energy of binding or were not significantly
381 different in peptide backbone position from other docking
382 poses, and thus were not considered for molecular dynamics.
383 Of the 15 peptide–protease complexes simulated, 12 were
384 found to have negative free energies of binding calculated by
385 the MM-PBSA approach. WSRVGYW was found to have a
386 favorable free energy of binding, and bound most strongly in
387 the active site. This is in good agreement with experimental
388 data for WSRVGYW.

389 Binding of LLEYSL in the active site is characterized by
390 hydrogen bonding of Asp30' and Asp25' to the Ser side chain
391 and second Leu backbone in the peptide sequence. These
392 bonds are present in all simulations where there was a favorable
393 free energy of binding for this peptide. The strongest predicted
394 binding structures also show hydrophobic interactions between
395 the peptide Tyr and Phe53'. The interaction of WSRVGYW
396 with the active site also shows significant dependence on
397 hydrogen bonding between the N-terminus Trp backbone and
398 Ser side chain with Asp30'. In these simulations, we also
399 observed several hydrophobic interactions between Tyr and the
400 C-terminus Trp with the Phe53 and Phe53' flap residues,
401 similar to that seen for LLEYSL.

402 We hypothesized that binding to the hinge region of the
403 protease could have a potential influence on the activity of the
404 enzyme, since an interaction here would prevent the flaps of the
405 enzyme from closing, hence preventing the formation of the
406 catalytic triad required for proteolysis. However, the
407 simulations showed that the binding of both WSRVGYW and
408 LLEYSL in this region was predicted to be orders of magnitude
409 less than in the active site. Binding conformations that originate

410 in the hinge region progressively interacted with the flaps over
411 the course of the simulations. Because of this only a few
412 hydrophobic interactions are observed between the peptides
413 and the Ile residues found in the protease flaps (Ile50, Ile54).
414 This is to be expected given that this is the hydrophilic, solvent-
415 exposed surface of the protease. From these studies it is
416 therefore reasonable to suggest that both WSRVGYW and
417 LLEYSL occupy the active site of HIV-1PR.

418 The interactions of the peptide sensors with the protease
419 were also simulated to observe the impact of the presence of
420 the dye molecule on the binding. Conformational sampling was
421 performed using the REST (replica exchange with solute
422 tempering) procedure with convergence determined by a
423 plateau in the number of new structures found by the clustering
424 analysis.

425 Clustering was performed on the two peptide backbones
426 separately with an RMSD cutoff of 1.2 Å over the final 50 ns of
427 the 200 ns REST trajectory (Figure 4a). The most populated
428 clusters found for both WSRVGYW and LLEYSL are
429 structurally similar to their respective conformations obtained
430 from the 800 ns classical molecular dynamics simulations. Both
431 peptides are relatively stable in their binding positions,
432 characterized by the change in free energy as a function of
433 the peptide radial distance from the HIV-1 PR catalytic residues
434 (Supporting Information, Figure S7). An enhanced discussion
435 of the clustering is given in the Supporting Information.

436 Interestingly, there are similar features between the binding
437 interactions of the two peptides (Figure 4b). Both sensors form
438 a hydrogen bond with the Gly48 backbone of the protease flap,
439 and both have persistent hydrophobic contacts with the
440 hydrophobic pocket formed by Ile50, Val82, Ile84, and
441 Leu23, as well as hydrophobic contacts with Phe53 on the
442 protease flap. The experimentally observed inhibitory action of
443 both peptides may be due to interference with the opening of
444 the flaps rather than occupation of the active site as seen with
445 other inhibitors.²⁹

446 To demonstrate the potential of this technique for one-step
447 diagnostics and to test whether our designed peptide sensors
448 generated a high efficiency signal, the FRET assay was
449 performed with the enzyme free in a buffered solution. In
450 this case the peptides were synthesized with fluorescein and
451 rhodamine conjugated to the N-terminus. These were
452 designated LLE-fluor and WSR-rhod, respectively. This was
453 done to modulate the fluorescence intensity of the rhodamine

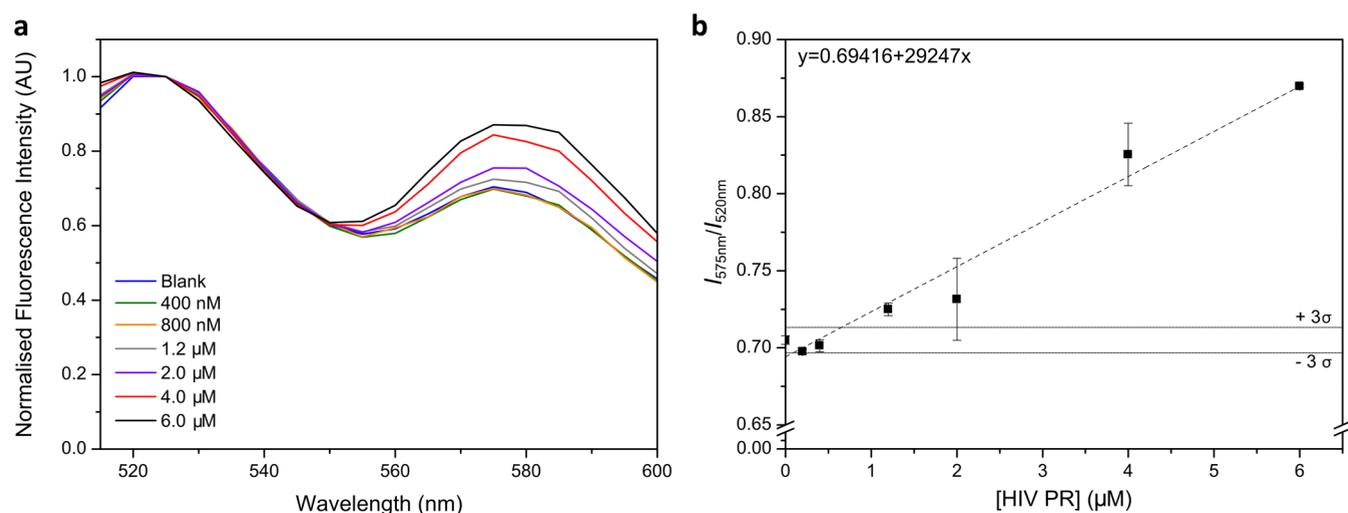


Figure 5. (a) Steady-state emission spectra of FRET in solution showing an increasing fluorescence transfer with increasing concentrations of HIV-1 PR ($\lambda_{ex} = 480$ nm) following a 1 h incubation at 4 °C. (b) Variation of the FRET signal with the concentration of HIV PR showing the limit of detection calculated as 3σ .

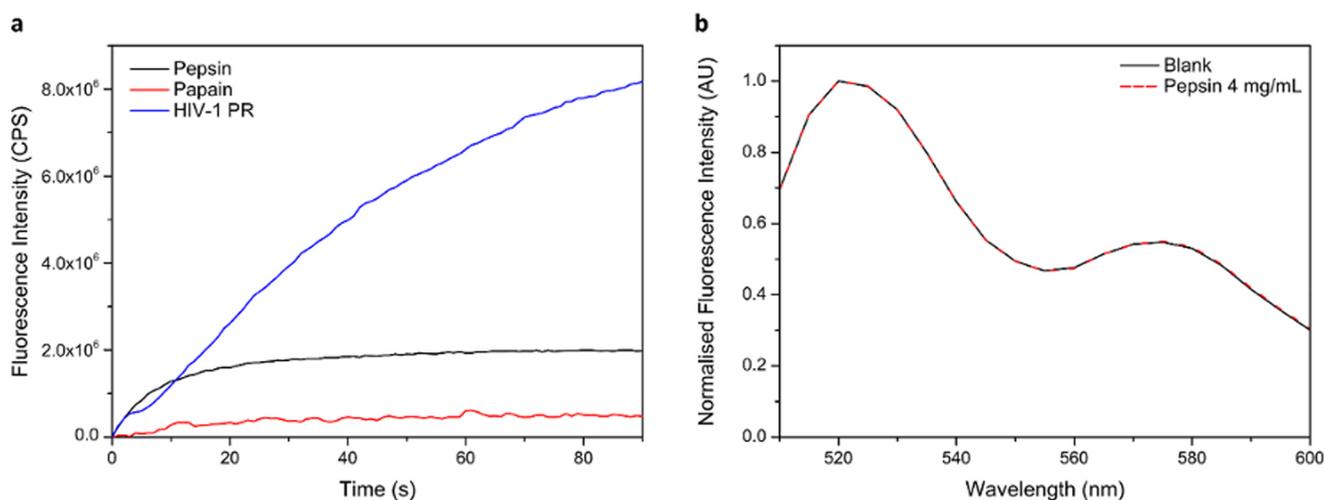


Figure 6. Comparison of assay specificity using different enzymes: (a) The commercial HIV-1 substrate shows some vulnerability to nonspecific cleavage by pepsin, an aspartyl protease but not to enzymes from a different family. (b) The assay developed here shows no response to an excess of pepsin when compared to the blank control.

454 which is highly pH dependent and more sensitive to cross-
 455 excitation at lower pH.³⁰ At this pH the protease is less active
 456 but not conformationally affected since its high pI means it is
 457 most stable between pH 4 and 6.³¹ HIV-1 PR was incubated
 458 with both peptide sensors at a concentration of 20 μM (LLE-
 459 fluor and WSR-rhod) for 1 h in the dark at 4 °C. The assay was
 460 incubated in the cold in order to slow the K_{off} rate of the
 461 peptides and thus improve the assay's sensitivity.

462 The FRET assay has a limit of detection of 654 nM
 463 calculated as the concentration of analyte that yields a signal
 464 higher than 3 times the standard deviation on the blank, 3σ
 465 (Figure 5). This is in good agreement with the lowest
 466 concentration which is observable by eye in the immobilized
 467 experiments (Supporting Information, Figure S4). In order to
 468 understand the low limit of detection observed for this assay,
 469 the FRET efficiency was calculated using time correlated single
 470 photon counting spectroscopy (TCSPC) in the presence of 4
 471 μM HIV-1 PR (Supporting Information, Figure S8).

The fluorescence lifetime of the donor molecule (fluorescein) in the presence and absence of the acceptor (rhodamine) was measured. In the absence of the acceptor, a single exponential gives a lifetime of 3.92 ns. In the presence of the acceptor the data follows a single-exponential yielding a decay of 3.53 ns. From this, using eq 1 where τ_D is the donor fluorescence in the absence of acceptor and τ_{DA} is the fluorescence of the donor in the presence of an acceptor, we can calculate an efficiency of only 9.95%.
 480

$$E = 1 - \frac{\tau_{DA}}{\tau_D} \quad (1) \quad 481$$

This low efficiency could be explained by several factors. First, it is possible that WSR-rhod is interacting with the enzyme further from the active site than is predicted by the models above (the simulations suggest a center-of-mass to center-of-mass distance between the two dye molecules of 2.68 (±0.27) nm). Second there is likely a dominant effect of the high k_{off} of WSR-rhod.
 488

489 Although we do not see two populations in the lifetime
490 measurements that would indicate the presence of an
491 unquenched population, the low rate of energy transfer may
492 mask this effect. Since the concentration of WSR-rhod used in
493 this assay is below the K_D of the peptide we can assume that at
494 this concentration only 30.9% of PR molecules are labeled with
495 an acceptor, while >99.98% of molecules will be labeled with a
496 donor molecule.

497 Calculating a distance between a FRET pair is difficult with
498 such a low efficiency since the r^6 dependence becomes
499 insensitive to changes above the Förster distance. Differences
500 between the calculated distance from the FRET studies (7.60
501 nm) and that expected from the MD may also arise due to the
502 different structure of the dye molecules which act as a reporter
503 probe (AF647/BHQ-3 vs fluorescein/rhodamine) which may
504 affect the calculated binding orientation, leading to differences
505 in center-of-mass distances. Other differences may occur due to
506 variations of the orientation factor (κ is assumed to be $2/3$ when
507 the dyes are free to rotate). Differences in this value would
508 impact the Förster radius used to calculate the dye–dye
509 distance. The use of rhodamine as an acceptor dye can also
510 introduce errors to this calculation due to its broad absorption
511 spectrum and hence significant direct excitation.

512 A major advantage of the proposed detection strategy
513 compared to classical approaches based on cleavable peptide
514 substrates is the high specificity of the detection due to the
515 multiple interaction events. In a conventional assay, proteases
516 with similar specificity to the target protease can also cleave
517 peptides with FRET pairs leading to false positives. Our test
518 does not rely on substrate cleaving and requires two
519 independent biorecognition events, which increases the
520 specificity of the assay. To prove the specificity of the proposed
521 detection scheme, the assay was repeated using pepsin. Pepsin
522 is the most ubiquitous of the aspartyl proteases and
523 demonstrates a broad substrate recognition.

524 Pepsin at the manufacturer's recommended working
525 concentration of 4 mg/mL was incubated at pH 2 (the optimal
526 pH for pepsin activity) with the commercial HIV-1 protease
527 substrate used for inhibition studies above. This acts as a model
528 system of a FRET pair linked through a cleavable peptide
529 substrate.

530 **Figure 6a** shows that the commercial system is vulnerable to
531 nonspecific cleavage since it yields a positive signal in the
532 presence of pepsin. The enzyme papain, a cysteine protease, is
533 included as a control from a different enzymatic family to show
534 that substrates are usually vulnerable to nonspecific enzymes
535 from structurally similar enzymes. In comparison, **Figure 6b**
536 shows the same concentration of pepsin (at large excess)
537 incubated with the peptide sensors. The experimental
538 conditions are identical to those used for the in-solution
539 FRET assay described above. In this instance, no difference can
540 be observed between the blank and the pepsin therefore
541 confirming our hypothesis that using two peptide sensors
542 results in highly specific signals.

543 ■ CONCLUSIONS

544 We have demonstrated that HIV-1 protease, a critical enzyme
545 in the lifecycle of HIV/AIDS, can be detected through designed
546 interactions with multiple peptide sensors. This approach to
547 biomarker detection requires two independent binding events,
548 a strategy that improves the specificity of the assay. Our
549 methodology is also differentiated from other protease-based
550 assays in that the signal is not dependent on proteolytic

cleavage which can be susceptible to nonspecific enzymes. We
551 also remove the need for the multiple wash steps that are
552 required with conventional ELISA assays, streamlining the
553 laboratory process. While the affinity of the peptide sensor
554 found through phage display to HIV-1 PR is lower than would
555 be expected from an antibody, we believe the advantages in
556 environmental stability and length scale make these sensors
557 well-suited to FRET applications. Through molecular dynamics
558 simulations we have explored the interaction between the
559 protease and the peptides to understand the mechanisms which
560 cause these binding events and developed a model for the
561 simultaneous binding of both peptide sensors to HIV-1
562 protease. It is hoped that our assay will provide a new direction
563 for the design of highly specific protein sensing without the
564 need for costly antibody development. 565

■ ASSOCIATED CONTENT

📄 Supporting Information

The Supporting Information is available free of charge on the
567 ACS Publications website at DOI: 10.1021/acs.chemmater.5b03651. 569

Additional figures showing supporting data, details about
571 calculations, and supporting methods details (PDF) 572

■ AUTHOR INFORMATION

Corresponding Authors

*Contact for modeling details. E-mail: irene.yarovsky@rmit.
574 edu.au. 575

*Contact for experimental details. E-mail: m.stevens@imperial.
577 ac.uk. 578

Present Address

[§]Department of Pure and Applied Chemistry, University of
580 Strathclyde, 295 Cathedral Street, Glasgow G1 1XL, United
581 Kingdom. 582

Author Contributions

All authors have given approval to the final version of the
584 manuscript. 585

Funding

The RMIT Foundation International Research Exchange
587 Fellowship is gratefully acknowledged for providing funds for
588 research exchange visits between Imperial College London and
589 RMIT University, Melbourne (K.-L.H). Australian Research
590 Council Discovery Grant DP 140101888 (I.Y. and M.M.S.) is
591 acknowledged. M.M.S. acknowledges EPSRC grant EP/
592 K020641/1 and the ERC Seventh Framework Programme
593 Consolidator Grant "Naturale CG" under grant agreement no.
594 616417. 595

Notes

The authors declare no competing financial interest. 597

■ ACKNOWLEDGMENTS

The authors thank Dr. Lisa Haigh (Department of Chemistry,
599 Imperial College London) for assistance with mass spectrom-
600 etry, Dr. Simon Burbidge of the Imperial College High
601 Performance Computing Centre for conversations regarding
602 these simulations, Dr. Nadav Amdursky (Imperial College
603 London) for assistance with TCSPC measurements, and Dr.
604 Philip Howes (Imperial College London) and Dr. Nevena
605 Todorova (Health Innovations Research Institute, RMIT
606 University) for useful discussions. We acknowledge the
607 generous allocation of high performance computational 608

609 resources from the Australian National Computational Infra-
610 structure (NCI), the Western Australian Computational
611 Facility (iVEC), the Victorian Partnership for Advanced
612 Computing (VPAC), and the Victorian Life Sciences Computa-
613 tional Initiative (VLSCI).

614 ■ ABBREVIATIONS

615 EDC, 1-ethyl-3-(3-(dimethylamino)propyl)carbodiimide;
616 NHS, *N*-hydroxysuccinimide; EDANS, 5-(2-
617 (aminoethylamino)-1-naphthalenesulfonate; DABCYL, 4,4'-di-
618 methylaminoazobenzene-4-carboxylate; HPLC, high pressure
619 liquid chromatography; BHQ-3, black hole quencher-3; AF647,
620 Alexa Fluor 647; HIV-1 PR, human immunodeficiency virus-1
621 protease; AIDS, auto immune deficiency syndrome; FRET,
622 Förster resonance energy transfer; Xgal, 5-bromo-4-chloro-3-
623 indoyl-*b*-D-galactoside; IPTG, isopropyl-*b*-D-thiogalactoside;
624 TMB, tetramethylbenzidine; TFA, trifluoroacetic acid; DMF,
625 dimethylformamide; DIPEA, *N,N*-diisopropylethylamine; MM-
626 PBSA, molecular mechanics-Poisson-Boltzmann surface area;
627 REST, replica exchange with solute tempering

628 ■ REFERENCES

629 (1) World Health Organisation, Global Summary of the AIDS
630 Epidemic 2013, 2014, accessed online [http://www.who.int/hiv/data/](http://www.who.int/hiv/data/en/)
631 [en/](http://www.who.int/hiv/data/en/).
632 (2) Navia, M. A.; Fitzgerald, P. M.; McKeever, B. M.; Leu, C. T.;
633 Heimbach, J. C.; Herber, W. K.; Sigal, I. S.; Darke, P. L.; Springer, J. P.
634 Three-dimensional Structure of Aspartyl Protease from Human
635 Immunodeficiency Virus HIV-1. *Nature* **1989**, *337*, 615–620.
636 (3) Pokorná, J.; Machala, L.; Rezáčová, P.; Konvalinka, J. Current and
637 Novel Inhibitors of HIV Protease. *Viruses* **2009**, *1*, 1209–1239.
638 (4) Mahmoud, K. A.; Hrapovic, S.; Luong, J. H. Picomolar Detection
639 of Protease Using Peptide/Single-Walled Carbon Nanotube/Gold
640 Nanoparticle-Modified Electrode. *ACS Nano* **2008**, *2*, 1051–1057.
641 (5) Mahmoud, K. A.; Luong, J. H. A Sensitive Electrochemical Assay
642 for Early Detection of HIV-1 Protease Using Ferrocene-Peptide
643 Conjugate/Au Nanoparticle/Single Walled Carbon Nanotube Modi-
644 fied Electrode. *Anal. Lett.* **2010**, *43*, 1680–1687.
645 (6) Choi, Y.; Lee, J.; Kim, K.; Kim, H.; Sommer, P.; Song, R.
646 Fluorogenic Assay and Live Cell Imaging of HIV-1 Protease Activity
647 Using Acid-Stable Quantum Dot-Peptide Complex. *Chem. Commun.*
648 (*Cambridge, U. K.*) **2010**, *46*, 9146–9148.
649 (7) Esseghaier, C.; Ng, A.; Zourob, M. A Novel and Rapid Assay for
650 HIV-1 Protease Detection Using Magnetic Bead Mediation. *Biosens.*
651 *Bioelectron.* **2013**, *41*, 335–341.
652 (8) Wong, E. Y.; Hewlett, I. K. HIV Diagnostics: Challenges and
653 Opportunities. *HIV Ther.* **2010**, *4*, 399–412.
654 (9) Suaifan, G. A.; Shehadeh, M.; Al-Ijel, H.; Ng, A.; Zourob, M.
655 Recent Progress in Prostate-Specific Antigen and HIV Proteases
656 Detection. *Expert Rev. Mol. Diagn.* **2013**, *13*, 707–718.
657 (10) Cornett, J. K.; Kirn, T. J. Laboratory Diagnosis of HIV in
658 Adults: A Review of Current Methods. *Clin. Infect. Dis.* **2013**, *57*, 712–
659 718.
660 (11) Medintz, I. L.; Clapp, A. R.; Brunel, F. M.; Tiefenbrunn, T.;
661 Uyeda, H. T.; Chang, E. L.; Deschamps, J. R.; Dawson, P. E.;
662 Mattoussi, H. Proteolytic Activity Monitored by Fluorescence
663 Resonance Energy Transfer Through Quantum-Dot-Peptide Con-
664 jugates. *Nat. Mater.* **2006**, *5*, 581–589.
665 (12) Lowe, S. B.; Dick, J. A. G.; Cohen, B. E.; Stevens, M. M.
666 Multiplex Sensing of Protease and Kinase Enzyme Activity via
667 Orthogonal Coupling of Quantum Dot-Peptide Conjugates. *ACS*
668 *Nano* **2012**, *6*, 851–857.
669 (13) Ghadiali, J. E.; Lowe, S. B.; Stevens, M. M. Quantum-dot-Based
670 FRET Detection of Histone Acetyltransferase Activity. *Angew. Chem.*
671 *Int. Ed.* **2011**, *50*, 3417–3420.

(14) Lee, T. G.; Maruyama, S. Isolation of HIV-1 Protease-Inhibiting
672 Peptides from Thermolysin Hydrolysate of Oyster Proteins. *Biochem.*
673 *Biophys. Res. Commun.* **1998**, *253*, 604–608.
674 (15) Artzy-Schnirman, A.; Abu-Shah, E.; Dishon, M.; Soifer, H.;
675 Sivan, Y.; Reiter, Y.; Benhar, I.; Sivan, U. On the Limited Recognition
676 of Inorganic Surfaces by Short Peptides Compared with Antibodies. *J.*
677 *Pept. Sci.* **2014**, *20*, 446–450.
678 (16) Molek, P.; Strukelj, B.; Bratkovic, T. Peptide Phage Display as a
679 Tool For Drug Discovery: Targeting Membrane Receptors. *Molecules*
680 **2011**, *16*, 857–887.
681 (17) Lee, C. V.; Liang, W.-C.; Dennis, M. S.; Eigenbrot, C.; Sidhu, S.
682 S.; Fuh, G. High-Affinity Human Antibodies from Phage-Displayed
683 Synthetic Fab Libraries with a Single Framework Scaffold. *J. Mol. Biol.*
684 **2004**, *340*, 1073–1093.
685 (18) Qi, H.; Qiu, X.; Xie, D.; Ling, C.; Gao, Q.; Zhang, C.
686 Ultrasensitive Electrogenated Chemiluminescence Peptide-Based
687 Method for the Determination of Cardiac Troponin I Incorporating
688 Amplification of Signal Reagent-Encapsulated Liposomes. *Anal. Chem.*
689 **2013**, *85*, 3886–3894.
690 (19) de la Rica, R.; Matsui, H. Applications of Peptide and Protein-
691 Based Materials in Bionanotechnology. *Chem. Soc. Rev.* **2010**, *39*,
692 3499–3509.
693 (20) Ph.D. Phage Display Libraries, New England Biolabs, accessed
694 online, June 2015, [https://www.neb.com/~media/Catalog/All-](https://www.neb.com/~media/Catalog/All-Products/A597C3819D8F421782485716A5AB8AD2/Datacards%20or%20Manuals/manualE8102.pdf)
695 [Products/A597C3819D8F421782485716A5AB8AD2/](https://www.neb.com/~media/Catalog/All-Products/A597C3819D8F421782485716A5AB8AD2/Datacards%20or%20Manuals/manualE8102.pdf)
696 [Datacards%20or%20Manuals/manualE8102.pdf](https://www.neb.com/~media/Catalog/All-Products/A597C3819D8F421782485716A5AB8AD2/Datacards%20or%20Manuals/manualE8102.pdf).
697 (21) Matayoshi, E.; Wang, G. T.; Krafft, G. A.; Erickson, J. Novel
698 Fluorogenic Substrates for Assaying Retroviral Proteases by Resonance
699 Energy Transfer. *Science* **1990**, *247*, 954–958.
700 (22) Esteban, A.; Popp, M. W.; Vyas, V. K.; Strijbis, K.; Ploegh, H. L.;
701 Fink, G. R. Fungal Recognition is Mediated by the Association of
702 Dectin-1 and Galectin-3 in Macrophages. *Proc. Natl. Acad. Sci. U. S. A.*
703 **2011**, *108*, 14270–14275.
704 (23) Morris, G. M.; Huey, R.; Lindstrom, W.; Sanner, M. F.; Belew,
705 R. K.; Goodsell, D. S.; Olson, A. J. AutoDock4 and AutoDockTools4:
706 Automated Docking With Selective Receptor Flexibility. *J. Comput.*
707 *Chem.* **2009**, *30*, 2785–2791.
708 (24) Case, D.; Darden, T.; Cheatham, T., III; Simmerling, C.; Wang,
709 J.; Duke, R.; Luo, R.; Walker, R.; Zhang, W.; Merz, K. AMBER 12;
710 University of California, San Francisco, 2012.
711 (25) Lindorff-Larsen, K.; Piana, S.; Palmo, K.; Maragakis, P.; Klepeis,
712 J. L.; Dror, R. O.; Shaw, D. E. Improved Side-Chain Torsion Potentials
713 For The Amber Ff99sb Protein Force Field. *Proteins: Struct., Funct.,*
714 *Genet.* **2010**, *78*, 1950–1958.
715 (26) Silva, A. M.; Cachau, R. E.; Sham, H. L.; Erickson, J. W.
716 Inhibition And Catalytic Mechanism Of HIV-1 Aspartic Protease. *J.*
717 *Mol. Biol.* **1996**, *255*, 321–346.
718 (27) Jorgensen, W. L.; Chandrasekhar, J.; Madura, J. D.; Impey, R.
719 W.; Klein, M. L. Comparison Of Simple Potential Functions For
720 Simulating Liquid Water. *J. Chem. Phys.* **1983**, *79*, 926–935.
721 (28) Ryckaert, J.-P.; Ciccotti, G.; Berendsen, H. J. C. Numerical
722 Integration Of The Cartesian Equations Of Motion Of A System With
723 Constraints: Molecular Dynamics Of N-Alkanes. *J. Comput. Phys.*
724 **1977**, *23*, 327–341.
725 (29) Wensing, A. M. J.; van Maarseveen, N. M.; Nijhuis, M. Fifteen
726 years of HIV Protease Inhibitors: raising the barrier to resistance.
727 *Antiviral Res.* **2010**, *85*, 59–74.
728 (30) Sjöback, R.; Nygren, J.; Kubista, M. Absorption and
729 Fluorescence Properties of Fluorescein. *Spectrochim. Acta, Part A*
730 **1995**, *51*, L7–L21.
731 (31) Szeltner, Z.; Polgar, L. Conformational Stability and Catalytic
732 Activity of HIV-1 Protease Are Both Enhanced at High Salt
733 Concentration. *J. Biol. Chem.* **1996**, *271*, 5458–5463.
734