Fluorescent-conjugated polymer superquenching facilitates highly sensitive detection of proteases

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Communicated by Thomas J. Meyer, Los Alamos National Laboratory, Los Alamos, NM, April 2, 2004 (received for review September 26, 2003)

Sensor formats have been developed for detecting the activity of proteolytic enzymes based on fluorescent conjugated polymer superquenching. These sensors employ a reactive peptide sequence within a tether linking a quencher to a biotin. The peptide binds to sensors containing colocated biotin-binding protein and fluorescent polymer by means of biotin–biotin binding protein interactions, resulting in a strong quenching of polymer fluorescence. Enzyme-mediated cleavage of the peptide results in a reversal of the fluorescence quenching. These assays for protease activity are simple, sensitive, fast, and have the specificity required for screening chemical libraries for novel protease inhibitors in a high-throughput screening assay environment. These assays have been demonstrated for enterokinase, caspase-3/7, and β-secretase.

Proteases play a key role in cell biology and have become priority targets for new pharmaceuticals (1). The interest in and the importance of the measurement of proteolytic activity is rapidly increasing for research, drug discovery and development, and as diagnostic or prognostic markers in disease states. For example, Alzheimer's disease is characterized by the extracellular deposition of insoluble amyloid plaques, which consist of a 4-kDa amyloid β-peptide (2). The amyloid β-peptide is derived from proteolysis of the amyloid precursor protein, a reaction catalyzed by β- and γ-secretases (3, 4). The enzyme β-secretase (BSEC) is essential for nerve cells to form the senile plaques (5–9). Most current therapeutic approaches to Alzheimer's disease involve finding drugs that block the BSEC catalytic site and disrupt its function.

Apoptosis (programmed cell death) plays a significant role in several disease states (6, 7). Because of a central role in inflammation and apoptosis, caspases (CASPs) have received enormous research attention (8). Results from nonspecific CASP inhibitors suggest that inhibition of CASPs could be sufficient to block apoptosis (9).

In view of the foregoing, it is important to develop rapid and sensitive assays for BSEC and CASP activities that can facilitate the rapid discovery of new drugs by using the high-throughput screening formats.

The QTL (quencher-tether-ligand) approach to biosensing takes advantage of the superquenching phenomenon of fluorescent polyelectrolytes by electron transfer and energy transfer quenchers (10–13). The phenomenon of superquenching involves association of a single electron or energy transfer quencher with a conjugated or dye-pendant polymer (or "polymer ensemble") and quenching of excitation delivered to any of a large number of polymer repeat units (PRU) (10–13). In one format, the fluorescent polymer P is colocated with receptors for a specific analyte on the surface of latex microspheres. The receptor can be attached to the microspheres by covalent linkage or by biotin–biotin binding protein (biotin–BBP) association. The assay is based on the competition for the receptor between the analyte and a synthetic QTL conjugate. For a successful direct competition assay, it is essential that the QTL conjugate associate with the receptor with a binding affinity comparable to that of the analyte. Although the fluorescence of the polymer-receptor ensemble is unaffected by the binding of the analyte, the fluorescence is quenched when the QTL is bound. Competition between the analyte and QTL for receptor sites is followed quantitatively by monitoring the fluorescence of the ensemble. Quantitative assays for small molecules and proteins have already been demonstrated by using the QTL technology (10, 14). In a second format for fluorescent polymer superquenching, a biotinylated anionic conjugated polymer is complexed to neutravidin (or another BBP) to form a solution sensor ensemble. The ensemble retains the biotin-binding sites and can associate through biotin–BBP interactions with a biotin quencher conjugate with concomitant quenching of the polymer fluorescence. This format has been shown to be effective as a platform for a peptide nucleic acid-based DNA assay (15, 16).

A sensor for protease enzymes was developed by incorporating a reactive peptide sequence within a tether linking the fluorescent polymer P with the quencher Q. We refer to this modification of the QTL approach as "QTP," where the QTP ensemble is a reactive molecular sensor that contains a quencher, Q, linked by a tether, T, which is recognized and cleaved by the target enzyme, to a fluorescent polymer P. The principle of this assay differs from the competition assays described above. In the absence of a specific association, or reaction of the QTP molecule with an enzyme or other target biomolecule, the fluorescence of P is attenuated or completely quenched by the proximity of Q. When the tether T is cleaved by the target enzyme, dissociation of the Q and P components is accompanied by a "turn-on" of polymer fluorescence. Fig. 1 shows the general scheme that is proposed for this assay. Because enzymatic cleavage of T is catalytic, amplification of the detection event occurs, providing a very sensitive assay for low levels of enzyme activity. In this paper, we report fluorescent polymer superquenching-based assays for three different proteases, namely, enterokinase (EK), CASP-3/7, and BSEC. We thus demonstrate that conjugated polymer superquenching provides a basis for platform technologies to develop rapid and sensitive assays for proteases.

Materials and Methods

Materials. The peptide substrates were custom synthesized at either Anaspec (San Jose, CA) or Bachem (Bubendorf, Switzerland) for the assay of proteases. The QSY-7 quencher was commercially obtained; the Azo quencher was synthesized by a routine procedure (17). The EK substrate is referred to as EK-1; substrates for BSEC are referred to as BSEC-1, BSEC-2, BSEC-3, and BSEC-4; and the CASP-3/7 substrate is CASP-3 (Fig. 2). Recombinant EK (light chain) enzyme (26.3 kDa) was purchased from New England Biolabs. Recombinant human CASP-3 and CASP-7 enzymes with a C-terminal histidine tag

Abbreviations: QTL, quencher-tether-ligand; BBP, biotin-binding protein; CASP, caspase; EK, enterokinase; PPE, *poly*(phenyleneethynylene); QTP, quencher-tether-polymer; QTB, quencher-tether-biotin; BSEC, β-secretase; biotin-RPE, biotin-R-phycoerythrin conjugate; PRU, polymer repeat unit.

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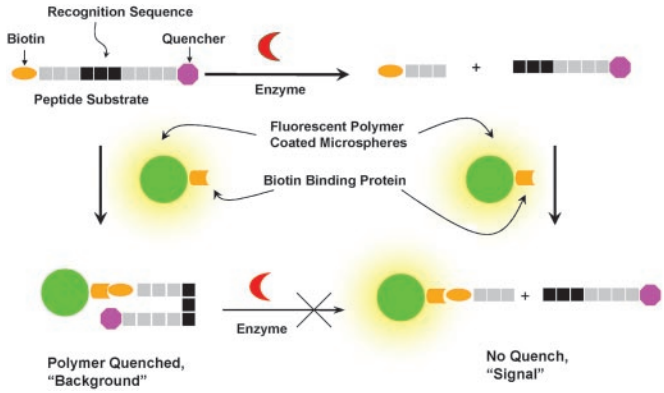

Fig. 1. General scheme for the QTL protease assay. This scheme shows microsphere-based polymer sensors A, B, and C. The solution sensors based on a polymer-avidin ensemble function in a similar manner.

were purchased from BD Biosciences. Recombinant human β-site amyloid precursor protein cleaving enzyme (73 kDa) was purchased from Enzyme Systems Products (Livermore, CA). The statine-derived BSEC inhibitor, STA-200, was also purchased from Enzyme Systems Products. Polystyrene microspheres used in the sensor preparations were purchased from

EK-1: (QSY7)-Lys-Ala-Gly-Ser-Gly-Ser-Glu-Asp-Asp-Asp-Asp-Lys↓Ala-Leu-Ala-Gly-Ala-Ser-Gly-(Lys-Biotin)-OH
(QSY7)-K-A-G-S-G-S-E-D-D-D-D-K-A-L-A-G-A-S-G-(K-Biotin)-OH

CASP-3: (AZO)-Asp-Glu-Val-Asp↓Gly-CO-NH-CH_2-CH_2-O-CH_2-CH_2-O-CH_2-CH_2-NH-Biotin
(AZO)-D-E-V-D-G-$(PEO)_3$-Biotin

BSEC-1: (QSY-7)-Thr-Glu-Glu-Ile-Ser-Glu-Val-Asn-Leu↓Asp-Ala-Glu-Phe-(Lys-Biotin)-OH
(QSY7)-T-E-E-I-S-E-V-N-L-D-A-E-F-(K-Biotin)-OH

BSEC-2: (QSY-7)-Thr-Glu-Glu-Ile-Ser-Glu-Val-Asn-Leu↓Asp-Ala-Glu-Phe-(Lys-PEG-Biotin)-OH
(QSY7)-T-E-E-I-S-E-V-N-L-D-A-E-F-(K-PEG-Biotin)-OH

BSEC-3: (AZO)-Thr-Glu-Glu-Ile-Ser-Glu-Val-Asn-Leu↓Asp-Ala-Glu-Phe-(Lys-Biotin)-OH
(AZO)-T-E-E-I-S-E-V-N-L-D-A-E-F-(K-Biotin)-OH

BSEC-4: (AZO)-Thr-Lys-Lys-Ile-Ser-Glu-Val-Asn-Leu↓Asp-Ala-Glu-Phe-Arg-(Lys-Biotin)-OH
(AZO)-T-K-K-I-S-E-V-N-L-D-A-E-F-R-(K-Biotin)-OH

STA-200: Lys-Thr-Glu-Glu-Ile-Ser-Glu-Val-Asn-(Statine)-Val-Ala-Glu-Phe-OH
K-T-E-E-I-S-E-V-N-(Statine)-V-A-E-F-OH

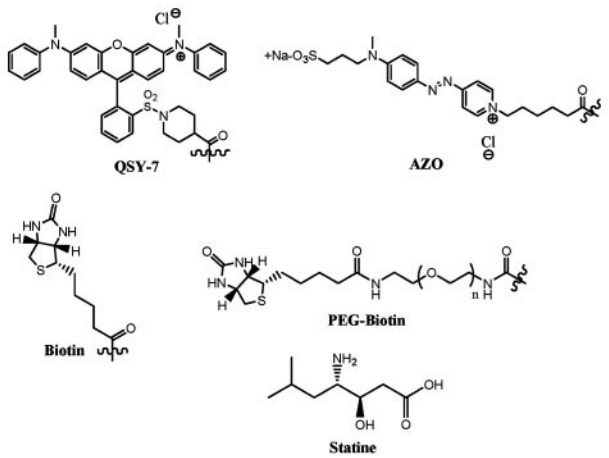

Fig. 2. Peptide substrates for EK, CASP-3/7, and BSEC and the BSEC inhibitor used in the QTL assays. EK-1 is the substrate recognized and cleaved by EK. Similarly, CASP-3 is a substrate for CASP-3 and CASP-7 enzymes, BSEC-1, BSEC-2, BSEC-3, and BSEC-4 are four substrates for BSEC. A downward arrow indicates the cleavage site for these peptides subject to enzymatic cleavage. STA-200 is a statine-derived peptide inhibitor of BSEC.

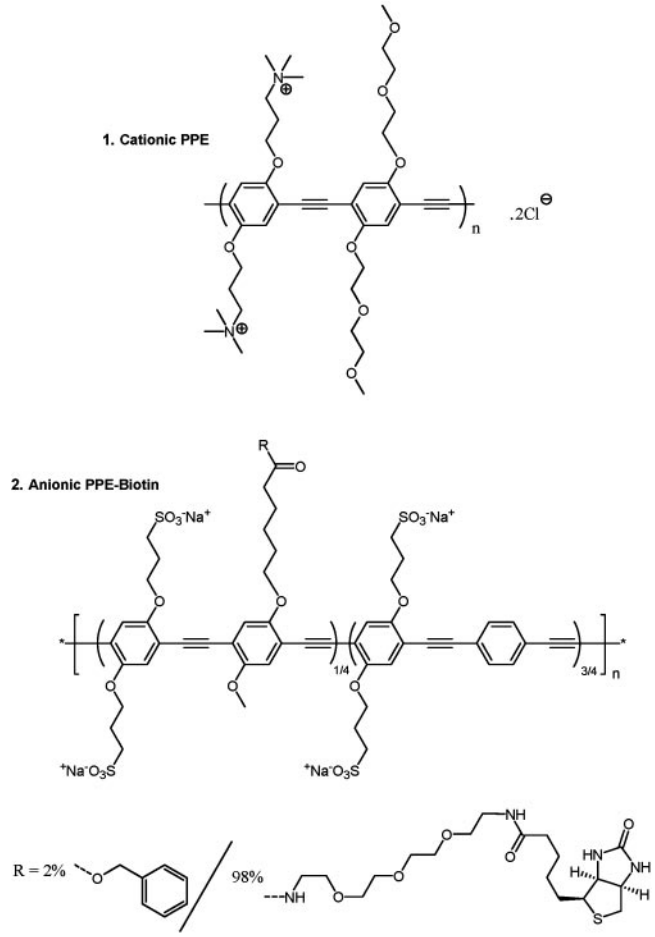

Fig. 3. Fluorescent-conjugated polymers used in the sensors.

Bangs Laboratories (Carmel, IN). Avidin and other BBPs were purchased from Pierce. Fluorescent *poly*(phenyleneethynylene) (PPE) polymers have been described earlier (15, 16). Matrix-assisted laser desorption ionization-time of flight analysis indicates the range of repeat units per polymer is ≈100–150.

Microsphere-Based Polymer Sensors. Microsphere-based polymer sensor A was prepared by mixing streptavidin-coated, 0.46-μm diameter, polystyrene latex microspheres with biotinylated anionic PPE (Fig. 3). The number of biotin binding sites available on a microsphere before polymer loading was estimated at 70,000. An 80 μM aqueous solution of biotinylated anionic PPE was added to 600 μl of streptavidin-coated microsphere at a concentration 1.85×10^{11} microspheres per ml, incubated for 4 h, and filtered through a 0.1-μm VVLP membrane in an ultrafiltration cell. Difference-fluorescence spectroscopy was used to quantify the polymer coating density at 129,000 PRU per microsphere. The number of biotin-binding sites per microsphere available to bind biotinylated fluorescein after polymer coating was determined to be 30,000.

To prepare microsphere-based sensor B, 36 μl of a 20 mM solution of a cationic conjugated polymer *poly*(*p*-phenyleneethynylene) (1) (Fig. 3), containing pendant ammonium groups at a level of two per PRU, was coated onto neutravidin BBP-derivatized polystyrene microsphere with a 0.6-μm diameter in a single step. Before coating polymer, the available biotin-binding sites were estimated to be 189,000 per microsphere. An aqueous solution of *poly*(*p*-phenyleneethynylene) was added to the microspheres, incubated for 4 h, and filtered through a

0.1-μm VVLP membrane in an ultrafiltration cell. Estimated polymer coating density was 1.05 million PRU per microsphere. The number of biotin-binding sites per microsphere accessible to biotinylated fluorescein after polymer coating was 22,600. An improved microsphere-based polymer sensor C was prepared by taking the microsphere-based polymer sensor described above and doping it with biotin-R-phycoerythrin conjugate (biotin-RPE) at a ratio of 1,500 molecules per microsphere. The phycoerythrin binds the microsphere by means of biotin–BBP interactions. Excitation of the PPE polymer at its absorption maximum at 440 nm leads to efficient energy transfer to the phycoerythrin chromophores and a sharp emission band at 576 nm. The sensor containing biotin-RPE (microsphere-based sensor C) provided the most sensitive assays thus far.

Solution Polymer Sensors. The solution polymer sensor was prepared by mixing together avidin (BBP) and biotinylated anionic PPE polymer (Fig. 3) in a ratio of 1 BBP:15 PRU and incubating at ≈25°C for 24 h. The solution sensor was then diluted with 100 mM phosphate buffer, pH 7.5, before use. As with the microsphere-based sensor, the solution polymer sensor was doped with biotin-RPE at a ratio of 1 molecule per 700 PRU to allow for sensitized emission from the avidin/PPE/biotin-RPE ensemble at 576 nm.

Methods. HPLC analysis of the peptides and reaction mixtures was performed on either a C8 or C18 reverse-phase column by using a mobile phase gradient of acetonitrile (0.1% trifluoroacetic acid), water (0.1% trifluoroacetic acid), and methanol.

The EK assay experiments were performed inside the wells of white BioPackard Optiwell 96-well plates (Perkin–Elmer). The assays were conducted in assay buffer (18) in a total volume of 14 μl on 29 μM peptide in polypropylene vials. After incubation with enzyme, an aliquot of the reaction mixture was diluted in water to 2 μM, and 5 μl of the diluted mixture was added to the microsphere suspension (2.0×10^7 microsphere-based polymer sensors per well in 90 μl of PBS). The fluorescence of the mixture was then measured.

BSEC and CASP-3/7 assays were conducted in a similar manner. The peptide substrates were incubated at ≈1 μM concentration in a typical reaction with the appropriate enzyme in 10 μl of assay buffer. The mixtures were incubated for 60 min at ≈25°C and the polymer sensor was added in either 20 or 40 μl of phosphate buffer. The assay and phosphate buffers were optimized for maximal speed and sensitivity of the enzymatic reaction. Fluorescence measurements were performed by using a SpectraMax Gemini XS plate reader (Molecular Devices) in well-scan mode at 440-nm excitation, with a 475-nm cut-off filter and either 530-nm emission for PPE-only sensors or 576-nm emission for PPE plus biotin-RPE-containing sensors.

Results and Discussion

As shown in Figs. 1 and 2, the assay was developed by using a synthetic peptide substrate as a tether containing the recognition and cleavage sequence and flanked by a nonfluorescent quencher (QSY-7 or Azo) and biotin at either termini. The peptide substrate is referred to as QTB (quencher-tether-biotin) and is associated with the polymer to form the QTP ensemble through biotin–BBP interactions. The EK, CASP-3, and BSEC enzymes have been shown to recognize and bind the peptide sequences DDDDK*A, DEVD*G and SEVNL*DAEF, respectively (16–19). After binding, the enzyme cleaves the amide bond at the site indicated by the asterisk. The QTB peptide substrates for the enzyme assay are thus designed to incorporate this sequence and are flanked by biotin on one end and a quencher on the other (Fig. 2). When the enzyme cleaves the appropriate peptide, the biotin-containing fragment and the quencher-containing fragment are separated. The quencher is thus left

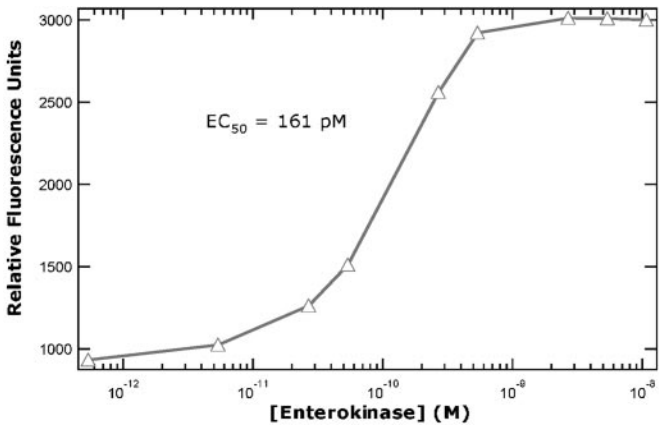

Fig. 4. Enzyme concentration response curve for EK. As low as 380 attomoles of EK was detected in a 15-min assay.

without a biotin and is unable to bind the polymer-receptor ensemble. Consequently, polymer fluorescence is restored after the enzyme catalysis.

In the initial assay, a functionalized polypeptide containing the amino acid sequence recognized by the protease enzyme EK (20) was designed with QSY-7 (nonfluorescent energy transfer quencher) covalently tethered to one end of the chain and a biotin at the other end. The basis for the assay is shown in Fig. 1. The synthetic "QTB" (quencher-tether-biotin) was predicted and found to bind to microspheres containing both cationic PPE (Fig. 3, polymer 1) and BBP with a concomitant quenching of the polymer fluorescence. It was further demonstrated that addition of the enzyme cleavage components (a peptide sequence linked to biotin and another peptide sequence linked to QSY-7) to the sensor produced only minimal quenching (probably by weak nonspecific association of the peptide QSY-7 residue with the microspheres). Moreover, mixtures of full and cleaved peptide fragments at various ratios when added to polymer at a constant concentration quenched polymer fluorescence in proportion to the amount of full peptide present in the mixture.

Initially, two types of experiments were carried out. In the first, a sensor mixture with the QTB bound to the fluorescent polymer-BBP ensemble (both microsphere-based and solution sensors, in separate experiments) was incubated with EK. In the second approach, the QTB was first incubated with the enzyme in solution, and then the reaction mixture was mixed with the fluorescent polymer-BBP ensemble. The first approach resulted in little change of fluorescence, possibly because of inhibition of QTB cleavage when anchored on the polymer microspheres. The absence of QTB cleavage was verified by HPLC analysis. However, the second approach provided a very sensitive assay for EK activity. Fig. 4 shows that polymer fluorescence increases with increasing concentration of enzyme and further that the amplification afforded by the catalytic cleavage of multiple QTB molecules by a single EK molecule provides the detection of extremely low concentrations of the enzyme. For example, a 15-min incubation of the enzyme with the QTB allowed for the detection of just 380 attomoles of EK. Incubation for longer periods will shift the curve shown in Fig. 4 to the left and permit detection of even lower amounts of the enzyme. The signal response was linear over an enzyme concentration range of nearly two orders of magnitude.

Four different peptide substrates were synthesized and used in the assay for BSEC. Whereas BSEC-1 and BSEC-2 contain QSY-7 as the quencher, BSEC-3 and BSEC-4 were labeled with azo dye as quencher. The BSEC-2 peptide also incorporated a long *poly*(ethylene glycol) tether. All four substrates bound to the

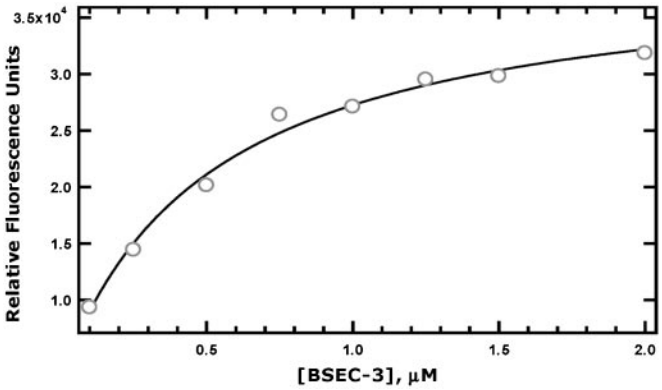

Fig. 5. Peptide substrate concentration response curve for BSEC-3 in the QTL assay. Relative fluorescence units were calculated from the difference of the fluorescence units measured for the sample containing substrate and enzyme and the control that contained substrate without enzyme.

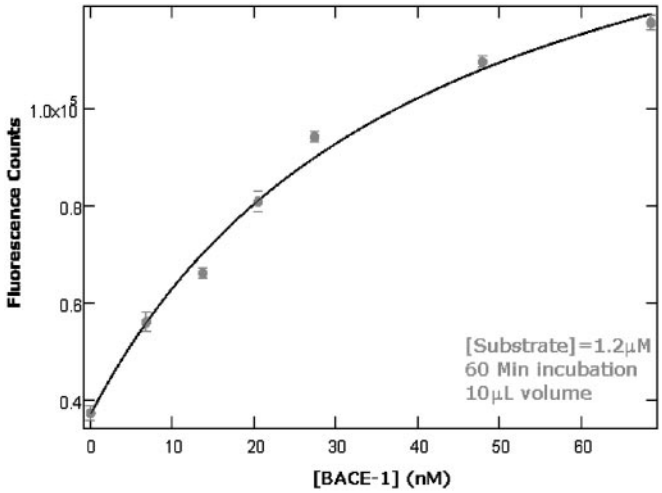

Fig. 6. Enzyme concentration response curve for BSEC (BACE-1) by using the QTL assay. Actual fluorescence counts measured are shown.

microsphere and solution polymer sensors through biotin–avidin binding interactions, and the fluorescence of the polymer was quenched. The fluorescence intensity of 5.0×10^6 microspheres of microsphere-based polymer sensor B was quenched ≈80% in the presence of 2 pmol of BSEC-1. The Stern–Volmer constant (K_{sv}) for BSEC-1 was 7.7×10^7 M^{-1} with 1.0×10^7 microspheres. The K_{sv} values for the other BSEC and CASP-3/7 peptides and polymer formats were found to be within the same range at similar concentrations of the polymer sensor (data not shown). The more relevant parameter (PRU per quencher at 50% quenching) is as high as 49 for CASP-3, indicating the role of superquenching in enhancing the sensitivity of these assays. However, as was observed for EK and EK-1, addition of enzyme even at large excess failed to hydrolyze the peptide substrate when bound to either microsphere or solution sensor (monitored by HPLC and fluorescence recovery experiments). It is possible that the enzyme is hindered from accessing the binding site on the peptide either through steric interactions with the polymer or by means of the irreversible adsorption of the enzyme to the hydrophobic polymer (21, 22). The introduction of a *poly*(ethylene glycol) functional group between the biotin and the peptide substrate was expected to improve the situation in one of the following ways: increase the distance between the cleavage site and the polymer to prevent steric hindrance to approach by the enzyme or reduce the hydrophobicity and, thus, reduce adsorption of enzyme to the polymer (23). The *poly*(ethylene glycol)-biotin labeled substrate (BSEC-2) bound to the polymer sensors and caused the expected quench response. However, incubation of these QTP ensembles did not lead to recovery of polymer fluorescence, suggesting that they were not effective enough in preventing either the adsorption of enzyme to polymer or the steric hindrance of polymer to the recognition of peptide by enzyme.

On the other hand, incubation of QTB molecules, such as BSEC-1 and CASP-3 with BSEC and CASP-3, followed by the addition of the microsphere or solution sensor resulted in a sensitive assay for enzyme activity. The peptide substrate and enzyme were incubated for 30 min in a total volume of 10 μl in BSEC assay buffer. The control sample contained the same amount of peptide without enzyme. After incubation, the sensor (microsphere- or solution-based) was added to the reaction mixtures, and the fluorescence intensity was measured. The amount of sensor to add per well was optimized to produce high relative fluorescence units to generate excellent signal intensity over background. The amount of peptide substrate in an assay was also optimized to provide efficient quench of polymer fluorescence and maximal "fluorescence recovery" in response to enzymatic activity. The data shown below were obtained with BSEC-3 (containing azo dye as quencher) at 150 nM in 10 μl of total reaction volume with BSEC enzyme, and a 40-μl suspension of microsphere-based sensor C containing 5×10^6 microspheres doped with 100 fmol of biotin-RPE (with the exception of the K_m experiment; see details below). Similar results were obtained with the other peptides for BSEC and other polymer formats (not shown here). The signal-to-noise values obtained in the assay at lower conversion rates is 15.

The K_m for BSEC-3 was determined from a QTP substrate concentration curve (Fig. 5). At the end of 30 min of incubation, microsphere-based sensor C (5×10^7 microspheres doped with 1 pmol of biotin-RPE per well) was added, and the quench responses were plotted as a function of substrate concentration. The K_m value was calculated to be 344 nM. This value is comparable to the K_m values reported in literature for BSEC substrates (24, 25).

Fig. 6 shows an increase in fluorescence intensity corresponding to increasing BSEC concentration. The assay allowed for the detection of BSEC activity within 5 min at concentrations as low as 13.7 nM. By increasing the catalysis duration, the limit of detection (limit of detection = blank relative fluorescence unit + 2 SD) improved significantly (Table 1). The QTL assay thus allowed for the detection of very low amounts of a relatively low-turnover enzyme, such as BSEC (k_{cat} = 0.02 sec^{-1}). The CASP-3 enzyme showed higher turnover and thus was detected in concentrations <1 nM.

The QTL assay for BSEC was also tested for interfering substances, such as DMSO, methanol, acetonitrile, colored compounds, inorganic salts, surfactants, and proteins. Significantly, the assay was tolerant of organic solvents and the other substances listed above when these were tested at concentrations normally encountered during high-throughput screening of drug candidates in the pharmaceutical industry. The inhibition of

Table 1. Limit of detection in BSEC enzyme kinetics

Reaction time, min	BSEC, fmol	BSEC, nM
5	137	13.7
10	116	11.6
20	84	8.4
30	48	4.8
60	13.7	1.4

BSEC activity in the presence of a known inhibitor of the enzyme was monitored by using the QTL assay. An IC_{50} value of ≈43 nM was obtained for STA-200 in a 30-min assay. This value is similar to the IC_{50} reported for this inhibitor with other reported assays that require longer incubation periods or higher concentrations of the enzyme or both.

In summary, the QTL assay for protease activity is general, simple, sensitive, fast, and has the required specificity for screening chemical libraries for novel inhibitors of enzyme activity in a high-throughput screening environment. [Peptide substrates designed to be cleaved by a specific protease (e.g., BSEC) have been shown to be unreactive toward other proteases (γ-secretase).] Moreover, the assay is robust, has been optimized for reactions in single tubes, 96-well, and 384-well plates, and can also be performed in 1,536-well plate format. The assay has been demonstrated for EK, CASP-3/7, and BSEC and was validated with a known selective inhibitor of BSEC.

We thank Tom Buscher, Mary Anne Burke, Kirsten Bradford, and Harshini Mukundan for technical assistance and Brent Burdick for helpful suggestions concerning the approach to an enzyme activity assay. This work was supported by the Defense Advanced Research Projects Agency under Contract MDA972-00-C-006.

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