HHS Public Access

Author manuscript

ACS Nano. Author manuscript; available in PMC 2017 June 28.

Published in final edited form as:

ACS Nano. 2016 June 28; 10(6): 6090–6099. doi:10.1021/acsnano.6b01682.

3,4-Dihydroxyphenylalanine Peptides as Non-Perturbative Quantum Dot Sensors of Aminopeptidase

Valle Palomo[1], **Sebastián A. Díaz**[2], **Michael H. Stewart**[3], **Kimihiro Susumu**[3], **Igor L. Medintz**[2], and **Philip E. Dawson**[1,*]

[1]Department of Chemistry, The Scripps Research Institute, La Jolla, California, 92037 (USA)

[2]Center for Bio/Molecular Science and Engineering, Code 6900, U.S. Naval Research Laboratory, Washington, D.C., 20375 (USA)

[3]Optical Sciences Division, Code 5611, U.S. Naval Research Laboratory, Washington, D.C., 20375 (USA)

Abstract

Fluorescence based assays for hydrolases that cleave within the substrate (endopeptidases) are common, while developing substrates for proteases that selectively cleave from peptide termini (exopeptidases) is more challenging, since the termini are specifically recognized by the enzyme and cannot be modified to facilitate a Förster resonance energy transfer (FRET)-based approach. The development of a robust system that enables the quenching of fluorescent particles by simple amino acid side chains would find broad utility for peptide sensors, and would be advantageous for exopeptidases. Here we describe a quantum dot (QD) based electron transfer (ET) sensor that is able to allow direct, quantitative monitoring of both exopeptidase and endopeptidase activity. The incorporation of 3,4-dihydroxyphenylalanine (DOPA) into the sequence of a peptide allows for the quenching of QD photoluminescence through an ET mechanism. DOPA is a non-proteinogenic amino acid that can replace a phenylalanine or tyrosine residue in a peptide sequence without severely altering structural properties, allowing for its introduction at multiple positions within a biologically active peptide substrate. Consequently, the quenching system presented here is ideally suited for incorporation into diverse peptide substrates for enzyme recognition, digestion and activity sensing. Our findings suggest a broad utility of a small ET-capable amino acid side chain in detecting enzyme activity through ET mediated QD luminescence quenching.

Graphical Abstract

Corresponding Author. Philip E. Dawson, dawson@scripps.edu.

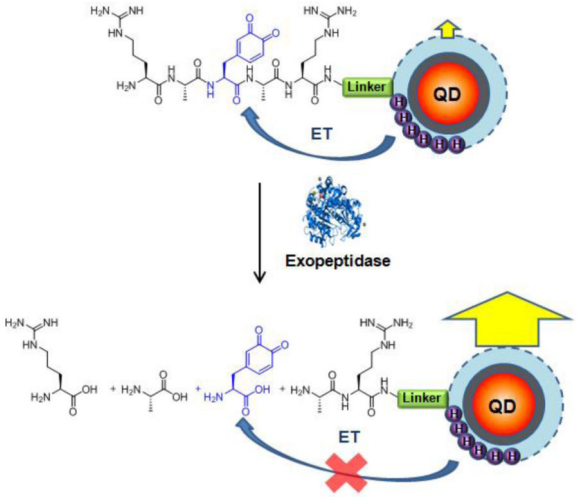
ASSOCIATED CONTENT

Supporting Information. Detailed materials and methods, photoluminescence data of SQQDS in 2 mM $Na_2B_4O_7$ at various pH values, peptide cleavage fragments data, photoluminescence data of SQQDs with peptides **1–3, 6, 7** before and after peptidase treatment and details of data analysis. This material is available free of charge *via* the Internet at http://pubs.acs.org.

The authors declare no competing financial interests.

Author Contributions

The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript.



Keywords

Quantum dot; nanoparticle; sensor; exopeptidase; DOPA; electron transfer

Enzyme activity is responsible for regulating chemical reactions in living organisms, and the alteration of specific enzyme activity can result in disease.[1] Understanding enzyme-substrate interactions is critical to comprehending their biological roles and to discovering new therapeutic targets and biomarkers.[2] Among enzymes, proteases account for approximately 2% of all proteins in humans and have been shown to play important roles in various physiological processes,[3] including coagulation, inflammation and myelination.[4,5,6] Exopeptidases are a subtype of the protease superfamily that selectively cleave substrates from termini. These enzymes are implicated in many relevant processes including pathogen activity,[7] lysosomal storage diseases and regulation of peptide hormones. Aminopeptidases are typically detected by the hydrolysis of peptide-aminocoumarin linkages, releasing a fluorescent aminocoumarin product. This approach has proven to be useful for fingerprinting of S1 substrate specificities[8] and in the development of diagnostic assays.[9] However, the positioning of the fluorophore is incompatible with amino acid sequences extending to the prime side of the cleavage site, that have been shown to be important elements of protease binding and catalysis.[10] The inherent limitations of activatable organic fluorophores such as aminocoumarin and the requirement for them to be incorporated at the scissile bond led us to consider other fluorescent molecules. Internally quenched Förster resonance energy transfer (FRET)-based peptide substrates have wide utility[11] but these substrates require modification of the termini in a manner incompatible with exopeptidases. Singly labeled peptide probes have also been developed, representing the advantage of using the peptide substrate as quencher, but the limitations of organic fluorophores as reporters and their susceptibility for aggregation inclined us to search for improved reporting systems.[12,13] We sought to develop an optical probe compatible with a variety of complex peptide substrates in a format that would be easy to assemble, have straightforward access to multiple colors, and be capable of dynamic signal change upon enzymatic processing. Previously, we developed a dopamine-based, quantum dot (QD) sensor for the monitoring of cellular pH[14]

that fulfilled much of our design criteria but is not sensitive to proteases. Since the redox active moiety of dopamine is also present in the amino acid 3,4-dihydroxyphenylalanine (DOPA), we reasoned that this amino acid could be utilized to quench photoluminescent QDs in a variety of peptide substrates including aminopeptidases.

Photoluminescent QDs represent a powerful platform for biological applications due to their narrow, tunable emission, high quantum yield, and pronounced photo and chemical stability.[15,16,17,18,19] For a fluorophore-quencher system, both the initial brightness of the fluorophore and quenching efficiency are critical for successful probe development.[20] QDs have demonstrated utility in bioanalytics as optical labels, facilitating a variety of QD-based biosensors,[21] and their efficiency for detecting protease activity has been confirmed.[22] Among other characteristics, their capacity to conjugate with multiple acceptor molecules and color tunability make them unique and powerful tools for bioimaging purposes.[23,24] The unique combination of QD photoluminescence quenching and complementary peptide substrates provides an ideal system to measure peptidase activity, because peptide substrates for proteases can be controllably self-assembled to QDs.[25] The most common approach for generating QD based protease sensors is through FRET. QD-FRET based nanosensors have been designed[26,27] and used as probes for proteases[28] for single and multiplexed tracking of enzyme activity,[29] for DNA and RNA detection[30,31,32] and for the development of biophotonic devices.[33,34]

The current format of a QD-quencher system is successful but limited. It generally requires a quenching dye that is covalently attached to the N- or C-terminus of the substrate. The substrate is then anchored to the QD, quenching its photoluminescence through proximity, leaving the fluorophore and quencher groups on either end of the enzyme's substrate. The quenchers used in energy transfer mechanisms are usually large organic molecules,[12, 35] and short distances between the donor molecule and the acceptor is frequently required, limiting the approach to short peptide sequences. A significant limitation to the FRET approach is that the chosen quencher may modify the structure and recognition of the peptide substrate due to its relatively large size. This fact is frequently overlooked and requires proper calibration in the sensors. In addition, this reporting method is poorly compatible with exopeptidases due to the terminal capping of the substrate[36,37,38] and therefore there is a need to develop an activity-sensing methodology with minimal perturbation of the substrate.

The incorporation of an amino acid analog within a peptide sequence that could act both as substrate and reporter of enzymatic activity would be an alternative option that eliminates an external element of the enzyme-substrate system. Ideally, such a method would allow unprotected peptides to act as both substrate and quencher, without requiring an additional quencher in the system. Accordingly, this approach would be of great benefit to measure exopeptidase or exoprotease activity (Figure 1).

Dopamine is a neurotransmitter that exhibits interesting redox properties[39] and can function as an electron acceptor in its quinone form. The effect of dopamine on QD luminescence or other fluorophores has been studied,[40,41] leading to the development of probes to measure intracellular pH,[14] tumor cell imaging,[42] and the detection of dopamine.[43] Dopamine exhibits a redox-active catechol ring that undergoes an increase in oxidation to quinone

proportional to pH increase. This quinone behaves as an electron acceptor of QDs when located near their surface, and quenches QDs photoluminescence. The ET based quenching of QD by dopamine, shared by the amino acid DOPA, opens alternative sensing possibilities that take advantage of a compact quencher. This amino acid that can be incorporated into substrates of proteases such as thrombin,[44] has been used in protein cross-linking,[45] and is a component of natural peptides such as mussel adhesives.[46] Although DOPA is an unnatural amino acid, the additional hydroxyl group is a small perturbation from Tyr/Phe and a growing body of work suggests that considerable structure variations in amino acid side chains can be readily accommodated by diverse proteases.[47] For example, aminopeptidases can exhibit a broad substrate tolerance including unnatural amino acids.[7,8] The ET strategy presented here is mechanistically distinct from FRET based strategies, facilitating the use of small donors or acceptors that better mimic amino acid side chains. Other ET strategies have utilized bulky external quenchers such as C_{60}[48] but the DOPA system allows for a much broader application in enzyme substrates.

Here we present a method using QD/DOPA peptide bioconjugates for peptidase activity sensing, utilizing the ability of DOPA to be positioned in the recognition site of a peptide (Figure 2). This strategy takes advantage of the QD quenching properties of DOPA to detect the hydrolytic activity of an exopeptidase, leucine amino peptidase (LAP, EC 3.4.11.1), and an endopeptidase, chymotrypsin, where the DOPA residue is an integral component of the peptide substrate. The approach is compatible with QDs coated with both neutral PEGylated and compact zwitterionic ligands. The gain in photoluminescence was robust and facilitated the kinetic characterization of LAP, which belongs to a class of enzymes that have been incompatible with previous luminescent nanoparticle systems.

RESULTS AND DISCUSSION

Design of DOPA-peptide substrates for self-assembly onto QDs

The peptides were designed in a modular manner encompassing a DOPA containing protease substrate, a polyproline (Pro_9) linker to ensure that the peptides are separated from the surface of the nanoparticle and accessible to the hydrolytic enzymes and a His-tag (His_6) sequence to facilitate self-assembly on the QD surface.[49] DOPA mediated quenching of QD photoluminescence has been shown to be distance dependent, yet still efficient using dihydrolipoic acid-PEG (DHLA-PEG) surface ligands of similar length to the Pro_9 linker used here.[50] Protease substrates contained a C-terminal Lys(aminooxy) (Lys(Aoa)) residue that enabled a convergent synthesis through an oxime ligation to the linker 4-formylbenzoyl-AhxPro_9TrpGlyHis_6 (Figure 3).[51] DOPA containing peptides were chain assembled by the Fmoc-protection strategy of solid phase peptide synthesis (SPPS) following procedures previously described.[52] The unnatural amino acid was presented primarily at the N-terminus of the sequence, however, as a non cleavable control, peptide **8** was synthesized with the DOPA residue at the C-terminus. To show the general utility of the DOPA quenching approach an alternate alpha helical sequence spacer for peptide **7** that was synthesized as a single peptide by Fmoc SPPS incorporating the substrate, the His-tag and the linker.

Quenching effect of DOPA-containing peptides and its variation with pH

Proteolytic enzymes are active over a range of pH values. To test for compatibility of the method and confirm the ET quenching mechanism, QD photoluminescence quenching by DOPA-containing peptides was studied at different pH values. The DOPA-containing peptides were self-assembled onto the QDs by mixing peptide **3** with DHLA-PEG coated QDs at ratios of 0, 15, 30, 45 and 60 peptides per QD for 30 minutes at room temperature. Previous results indicated a maximum of ~50–60 peptides could be stably assembled on to the surface to prepare QD-peptide conjugates.[53] Following conjugation, photoluminescence spectra scans were taken from 480 to 560 nm, exciting at 350 nm. The experiments were performed in 0.1×PBS at 5.5, 7.0 and 9.5 pH values. Figure 4 shows the photoluminescence quenching of the increasing DOPA peptide loading at pH 5.5, 7.0 and 9.5.

At each specific pH, the luminescence quenching (ratio I_0/I) is linearly dependent on peptide load, yielding lower luminescence with increasing peptide:QD ratios (Figure 4). For a given peptide:QD ratio, the degree of quenching significantly increases with basic pH due to the shifted equilibrium to the quinone quencher at higher pH values.[54] Figure 4D shows a Stern-Volmer plot comparing both peptide load and pH. Similar quenching trends were observed using 2 mM $Na_2B_4O_7$ buffer (Figure S1). This data suggests that the amino acid DOPA incorporated into a peptide substrate through a Pro_9WGHis_6 linker quenches QD photoluminescence through an ET mechanism, and is in agreement with previous studies of dopamine quenching of QDs.[50] Substrate quenched QDs (SQQDs) were assembled with all peptides described in Figure 3, using neutral DHLA-PEG-QDs and DHLA-CL4-QDs[55] that provides a compact zwitterionic net negatively charged QDs surface.

Analysis of peptide substrates cleavage in solution by LC-MS

Non-QD conjugated DOPA containing-peptides were incubated with chymotrypsin (endopeptidase) and LAP (exopeptidase), to confirm that these enzymes recognize the non-proteinogenic amino acid as a substrate. Since chymotrypsin recognizes the aromatic DOPA in the primary P1 recognition pocket, it represents a stringent test for the utility of DOPA in protease substrates. Peptide substrate RAXARK (90 μM) was dissolved in 2 mM $Na_2B_4O_7$ buffer at pH 8.5 and chymotrypsin was added to afford a final enzyme concentration of 0.4 μM. The mixture was incubated at 37 °C for 5 minutes and then analyzed by reversed-phase HPLC-MS to identify the cleavage products (Figure S2). RAX was the only fragment identified, consistent with recognition of DOPA by chymotrypsin in the P1 position.[56] This demonstrated that DOPA can be recognized and cleaved as an enzyme substrate, and is consistent with the known preference of chymotrypsin for hydrophobic amino acids.[57]

Similar experiments were performed using non-QD conjugated peptides **1–7** and LAP. Peptide **1** was dissolved in 2 mM $Na_2B_4O_7$ buffer at pH 8.5 at 110 μM, and LAP was added to afford a final enzyme concentration of 1.5 μM. The mixture was incubated at 37 °C for 5 minutes and then analyzed by reversed-phase HPLC-MS to identify the cleavage products. One sole fragment was identified, corresponding to a C-terminal fragment of peptide **1** lacking the entire substrate sequence RPXAR (Figure S3). This indicates that the substrate module of peptide **1** was digested from the N-terminus down to the oxime linker (Figure 3).

The entire peptide substrate, including the DOPA residue, was not observed, consistent with the sequential hydrolytic mechanism of LAP.[58]

At a lower LAP concentration of 0.05 μM, the incubation with peptide **1** yielded two products after enzyme digestion, corresponding to the fully intact peptide **1** and the digested C-terminal fragment observed previously. Similarly, peptides **2–6** yielded a sole C-terminal fragment corresponding to the linker without the DOPA when incubated with a lower LAP concentration (0.03–0.12μM). These findings suggest that LAP acts processively on the studied peptides **1–6**, which is consistent with LAP proccessive cleavage mechanism.[59] Interestingly, in the case of peptide **7** (same substrate sequence as **3**), when using a 0.036 μM of LAP, different C-terminus peptidic fragments were found, corresponding to the peptide individual single amino acid cleavage steps from the N-terminus (Figure S4). We interpret this data as resulting from a lack of processivity due to the intrinsic secondary structure induced by the unnatural amino acid aminoisobutyric acid (Aib)[60] in the proximal linker, which has been shown to avoid degradation in proteases.[61,62]

Altogether, the data collected from peptide digestions in solution suggest that the unnatural amino acid DOPA is being recognized by the hydrolytic enzymes chymotrypsin and LAP. For chymotrypsin, the DOPA residue was cleaved exclusively in position P1, and for LAP it was found that DOPA was recognized at the P1 and P1', P2' and P3'positions. These results suggest that DOPA can be widely utilized in peptidase substrates.

Chymotrypsin digestion of SQQDs

Peptides **1** and **3** were self assembled to DHLA-CL4-QDs. Following self-assembly of SQQDs in 2 mM $Na_2B_4O_7$ at pH 8.5, the QD bound peptides **1** and **3** were fully enzymatically hydrolyzed by adding chymotrypsin (1.6 μM) at 37 °C for 1 h. QD emission was recorded before and after enzyme digestion. Comparison of the emission spectra before and after enzyme treatment showed an increase in photoluminescent emission of the conjugated QDs after enzyme treatment (Figure 5). As expected for full hydrolysis of the quenching DOPA group, the recovery of photoluminescence was independent of the peptide:QD ratio, reaching emission values of the unconjugated control. Similar results were encountered using DHLA-PEG coated QDs with peptide **1** and DHLA-CL4 QDs with peptide **6** (Figure S5). Together, these findings confirm that DOPA peptides can be assembled on the surface of QDs and be recognized as substrates by hydrolytic enzymes and regain their photoluminescence.

Aminopeptidase digestion of SQQDs

Following self-assembly, DHLA-PEG-coated QDs bound peptides **1** and **8** were mixed with LAP (0.5 μM) at 37 °C for 1h. SQQDs with a cleavable substrate (**1**) showed an increase of photoluminescence after enzyme treatment, while with the non-cleavable substrate (**8**) they remained quenched after enzyme incubation, indicating that the DOPA-containing peptides remained bound to the surface of the QDs (Figure 6). The photoluminescence of the QD is recovered only after LAP cleavage of DOPA from the N-terminus. Similar results were obtained with other DOPA peptide substrates **2, 3** and **7** (Figure S6). LAP recognized all peptide constructs and the SQQDs gained a level of luminescence comparable to the control

without DOPA peptides. These findings reinforce the versatility of substrate and linker design in this system, and confirm that the method presented here is applicable to the detection of aminopeptidase activity.

Kinetic evaluation of SQQDs cleaved by LAP

The luminescence intensity of QDs is extremely high, and the expected K_m value of LAP is expected to be in the middle micromolar range. As a result, it is not possible to perform typical Michaelis-Menten kinetics with a true excess of substrate. Instead, the fixed substrate format was used to calculate the catalytic efficiency (k_{cat}/K_m) of LAP using SQQDS, following the method used previously for QD-FRET proteolytic assays.[63] DHLA-PEG-coated QDs were self-assembled with peptide **2** at a ratio of 1:15 and at a concentration of 0.25 μM for 30 minutes in $Na_2B_4O_7$ at pH 8.5. Following self-assembly, the SQQDs were incubated with LAP (0 nM; 1.62nM; 3.25 nM; 16.25 nM; 32.5 nM) and photoluminescence was monitored at 520 nm at 30 s intervals for 5220 s (87 min) using an excitation wavelength of 350 nm. Analysis similar to that in our previous work allowed for a kinetic analysis of LAP with SQQDs.[63,64] A calibration curve, consisting of an increasing ratio of QD/DOPA peptide 1:30, 1:8, 1:3 and 1:0, was built to allow the transformation of the progress curves (Figure 7) into cleavage of peptides on the surface of the nanoparticles. From the collected data, a catalytic efficiency was calculated for peptide **2** (k_{cat}/K_m=50 ± 15 mM^{-1} s^{-1}) based on the assumption that the system could be properly fit by a Michaelis-Menten mechanism, obtaining a value consistent with literature.[65] It is important to note that standard excess substrate formats can be used in QD systems when the K_m value of the enzyme/substrate in question is significantly lower, as described in our previous work.[66] An extended presentation of the kinetic methodology can be found in the SI.

CONCLUSION

We show that DOPA residues in peptides can simultaneously act as part of the peptide substrate while serving as QD quenchers. This methodology represents a flexible approach for the development of photoluminescent enzyme substrates that utilizes a simple quenching system that closely mimics the natural amino acid tyrosine, and is thus amenable to incorporation into a broad range of peptide substrates. The unnatural amino acid DOPA, when inserted into a peptide sequence has the ability to quench QDs through an ET mechanism, and its efficiency is dependent on both the number of peptides loaded on the nanoparticles and on the pH of the solution. The designed substrates quench QD luminescence at various pH values ranging from 5.5 to 9.5, thus covering the optimal pH of diverse proteases. The side chain of DOPA can be recognized in the primary P1 position of chymotrypsin. Importantly, since the N-terminus of the peptide can remain unmodified, DOPA peptides can be fully processed by exopeptidases such as LAP with high kinetic efficiency. The versatility and recognition shown in this study suggests that this method could be broadly applied to both endo- and exopeptidases with little alteration to the peptide sequence, in contrast to the bulky chromophores used for FRET based substrates. Together, these results show that QD/DOPA peptide bioconjugates constitute a robust and versatile method for reporting peptidase activity.

METHODS

Materials and Methods

All chemicals and solvents were purchased from commercial sources and used without further purification: DMF (Dimethylformamide, HPLC grade), CH_3CN and CH_2Cl_2 from Fisher, TFA (trifluoroacetic acid), DIEA (N,N-diisopropylethylamine) and 4-formyl-benzoic acid (4FB) from Sigma-Aldrich. Water was purified using a Millipore Milli-Q water purification system. HCTU (o-(1-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate) and HOBt (1-hydroxybenzotriazole) from Peptides International, HATU (o-(7-azabenzotriazol-1-yl)-1,1,3,3-tetramethyluroniumhexafluorophosphate) from Anaspec. Fmoc-L-DOPA(acetonide)-OH and Fmoc-6-aminohexanoic acid (Ahx) were purchased from Novabiochem and the rest of amino acids from Bachem. MBHA resin (4-methylbenzhydrylamine, 0.65 mmol/g) was purchased from Peptides International and Rink amide (0.68 mmol/g) from Novabiochem. Chymotrypsin and LAP were purchased from Sigma-Aldrich. TIS (Triisopropylsilane) was purchased from Oakwood Chemical. Analytical reverse phase HPLC was carried out in a Varian ProStar Model 210 equipped with a Dynamax Absorbance Rainin Detector. Analytical injections were monitored at 214 nm. Separations were performed using different columns and gradients detailed at every product. Preparative HPLC was performed in a Waters Delta Prep 4000 equipped with a Waters UV detector model 486 and a Phenomenex Jupiter Proteo column (10 μM, 90 Å, 250 × 21.20 mm) at a flow rate of 15 mL/min employing a gradient specified for each peptide. Preparative injections were monitored at 220 nm.

Peptides were characterized using electrospray ionization MS on a LC/MS API 2000 Plus triple quadrupole mass spectrometer (Sciex). Peptide masses were calculated from the experimental mass to charge (m/z) ratios from all observed protonation states by using the Analyst software (Sciex). UV measurements were undertaken in a Genesys 6 UV/vis spectrometer (Thermo Electron Corporation). Fluorescence recording for single samples was performed with an AVIV automated titrating differential/ratio spectrofluorometer Model ATF 105. Fluorescence recording for kinetic measurements was performed with a Tecan Infinite 200 Pro Fluorescence plate reader.

Peptide Synthesis

Polyproline peptides were chain assembled manually using *in situ* neutralization cycles for Boc-solid-phase-peptide synthesis (Boc-SPPS) following procedures described in the literature.[67] Peptides were synthesized on 0.2 mmol MBHA resin (4-Methylbenzhydrylamine, 0.65 mmol/g). Boc-amino acid (1.5mmol) was dissolved in 2.66 mL of 0.5 M HCTU in DMF (1.33mmol), and DIEA (2.66 mmol, 464 μL) was added. After 30 s, the solution was added to the resin. Coupling times were 20 min. For proline residues, the resin was neutralized with 10% DIEA in DMF (2 washes of 1.5 min each) after every TFA deprotection. Boc-proline (2.0 mmol, 430 mg) was dissolved in 4 mL of 0.5 M HCTU in DMF (2.0 mmol), and DIEA (2.0 mmol, 348 μL) was added. After 30 s, the solution was added to the resin. Double couplings were performed for 1 h. Following chain assembly, peptides were cleaved from the resin with HF and 10% of anisole for 1 h at 0 °C. After that time HF was evaporated and cold ether was added to precipitate the crude peptides. The

resin was filtered, and the crude was dissolved in 30% Buffer B (0.05% TFA, 90% CH_3CN, 10% H_2O) in Buffer A (0.05% TFA in H_2O) and lyophilized.

DOPA-containing peptides were chain assembled by manual Fmoc-SPPS, using 0.1 mmol Rink amide resin. Fmoc-amino acid (0.5 mmol) was dissolved in 1.25 mL of 0.4 M HCTU in DMF (0.5 mmol), and DIEA (0.75 mmol, 130 μL) was added. After 30 s, the solution was added to the resin. Coupling times were 25 min. Fmoc-Lys(ivDde)-OH (0.2 mmol, 115 g) was dissolved in 1 mL of 0.4 M HATU in DMF (0.2 mmol) and DIEA (0.3 mmol, 52 μL) was added. After 30 s, the solution was added to the resin. Coupling time was 40 min. Fmoc-L-DOPA(acetonide)-OH (0.15 mmol, 68 mg) was dissolved in 375 μL of 0.4 M HATU in DMF (0.15 mmol) and DIEA (0.225 mmol, 39 μL) was added. After 30 s, the solution was added to the resin. Coupling time was 1 h. Following chain assembly, the terminal Fmoc group was removed and Boc-anhydride (0.5 mmol, 109 mg) was dissolved in 0.5 mL of DMF and DIEA (0.5 mmol, 87 μL) was added. After 30 s, the solution was added to the resin. Coupling time was 30 min, and coupling efficiency was checked with a Kaiser test. Protecting group ivDde was removed with 4 washes of 4% hydrazine hydrate in DMF for 5 min. After deprotection of the lysine side chain the resin (0.05 mmol) was treated to incorporate the Aminooxy (Aoa) group. Boc-Aoa-OH (0.25 mmol, 47.8 mg) was dissolved in 0.5 mL of 0.5 M HOBT in DMF (0.25 mmol) and DIC (0.25 mmol, 38.6 μL) was added. After 30 s, the solution was added to the resin. Coupling time was 30 min, and coupling efficiency was checked with a Kaiser test. Peptides were cleaved from the resin using a cleavage cocktail that contained TFA (95%), TIS (2.5%) and H_2O (2.5%). Resins were treated with the cleavage cocktail for 120 min. After that time the resin was filtered and TFA was evaporated using a gentle stream of N_2 over the mixture. The crude peptides were precipitated with cold ether, and dissolved in 30% Buffer B (0.05% TFA, 90% CH_3CN, 10% H_2O) in Buffer A (0.05% TFA in H_2O) and lyophilized.

Synthesis of 4FB-Ahx-Pro_9TrpGlyHis_6

4-Formylbenzoyl (4FB) was coupled to Ahx-Pro_9TrpGly$_2$$His_6$ (5.3 mg, $2.6.10^{-3}$ mmol) in 25 mM NaH_2PO_4 buffer at pH 7.0 (2.7 mL) using succinimidyl-4-formylbenzoate (1.3 mg, $5.5.10^{-3}$ mmol, 2 eq.) for 3 h. The reaction mixture was purified by preparative HPLC using a gradient from 15 to 35% Buffer B (0.05% TFA, 90% CH_3CN, 10% H_2O) in Buffer A (0.05% TFA in H_2O) in 45 min. 4.2 mg of white solid (74%) were recovered after lyophilization.

General procedure for synthesis of peptides 1–6 and 8

1 eq. of 4FB-Ahx-Pro_9TrpGlyHis_6 was mixed with 2 eq. of DOPA-containing peptide in 50 mM $NH_4C_2H_3O_2$ buffer at pH 4.5 (keeping peptide concentrations at 0.5 mg/ml) for 1 h. The mixtures were then purified to afford the final peptides. A gradient from 5 to 30% Buffer B (0.05% TFA, 90% CH_3CN, 10% H_2O) in Buffer A (0.05% TFA in H_2O) during 45 min was used for purification. HPLC fractions with pure peptide were collected and lyophilized. After HPLC purification and lyophilization the peptides were obtained as white solids with the following recovered yields: **1** 95%; **2** 84%; **3** 93%; **4** 64%; **5** 97%; **6** 97%; **8** 97%.

QD Synthesis

523 nm emitting CdSe/ZnS core−shell QDs were synthesized according procedures previously reported with some modifications.[68,69] Following the synthesis, the original hydrophobic ligands were exchanged with DHLA-PEG$_{750}$[70] or DHLA-CL4.[55]

SQQDs assembly and fluorescent analysis at different pH values

DHLA-PEG-QDs (~0.5 μM) were incubated with peptide **3** (0.5–25 μM) at ratios of 0, 15, 30, 45 and 60 peptides per QD for 30 min at room temperature in 0.1×PBS buffer (250 μL total volume) at the pH value specified. The samples were excited at 350 nm, and fluorescence spectra were collected from 480 to 560 nm in an AVIV 105 fluorometer.

Peptide enzymatic assays

PBS and borate buffers were tested for enzyme incubations, where borate buffer was found to be the preferred choice for these studies. *Chymotrypsin digestion*. Peptide RAXARK in 2 mM $Na_2B_4O_7$ pH 8.5 buffer (6 μL, 112 μM) was incubated with chymotrypsin (1.5 μL, 2μM) (final concentrations of 89.6 μM of RAXARK and 0.4 μM chymotrypsin) at 37 °C during 5 min. After that time the mixture was analyzed by HPLC-MS. Fragment mass found: 425.1 $[m+1]^+$.*LAP digestion.* To afford complete enzymatic hydrolysis peptide **1** (65 μL, 269 μM) in 2 mM $Na_2B_4O_7$ buffer at pH 8.5 was incubated with LAP (15 μL, 6.5 μM) (final concentrations of 218.5 μM of **1** and 1.2 μM LAP) at 37 °C during 5 min. To partially hydrolyze the substrate, peptides **1** and **7** in 2mM $Na_2B_4O_7$ buffer at pH 8.5 (17 μL, 269 μM) and (25 μL, 18.6 μM) were incubated with LAP (1.5 μL, 0.65 μM) (final concentrations of 247.2 μM of **1** and 0.052 μM LAP; 17.54 μM of **7** and 0.036 μM LAP) at 37 °C during 5 min. Fragment mass found for **1**: 801.9 $[(m+3)/3]^+$, 1202.3 $[(m+2)/2]^+$. Fragment mass found for **7**: 738.9 $[(m+3)/3]^+$, 1107.7 $[(m+2)/2]^+$; 715.0 $[(m+3)/3]^+$, 1072.3 $[(m+2)/2]^+$; 655.6 $[(m+3)/3]^+$, 982.8 $[(m+2)/2]^+$; 631.8 $[(m+3)/3]^+$, 947.3 $[(m+2)/2]^+$; 869.2 $[(m+2)/2]^+$; 769.5 $[(m+2)/2]^+$.

SQQDs enzymatic assays

Chymotrypsin digestion—Peptides **1** and **3** were mixed with DHLA-CL4 QDs at ratios of 0, 10, 18 and 36 peptide load per QD. QD concentration was ~0.05 μM. Following self-assembly, an emission scan was recorded from 480 to 560 nm for each sample, exciting at 350 nm. The QD bound peptides were fully enzymatically hydrolyzed by adding chymotrypsin (1.6 μM) at 37 °C for 1 h and the QD emission was recorded again. *LAP digestion.* Peptides **1** and **8** were self-assembled to DHLA-PEG coated QDs for 30 min at room temperature in a 2 mM $Na_2B_4O_7$ buffer at pH 8.5 at ratios of 0, 10, 20 and 40 peptides per QD. During self-assembly, QD concentration was ~0.3 μM. LAP (0.5 μM) was added and the mixture was maintained at 37 °C for 1h, and the QD emission was recorded again.

Kinetic assays

DHLA-PEG-coated QDs (0.25 μM) were mixed with peptide **2** for 30 minutes at a ratio of 1:15 and at a concentration of 0.25 μM in a 2 mM $Na_2B_4O_7$ buffer at pH 8.5. After that time the conjugates were incubated with different amounts of LAP (0 nM; 1.62nM; 3.25 nM; 16.25 nM; 32.5 nM). Photoluminescence measurements were collected at 30 s intervals and

finished at 5220 s (87 min) using an excitation wavelength of 350 nm and an emission wavelength of 520 nm. Alongside this assay, a calibration curve was built containing SQQDs at QD:peptide ratios of 1:30, 1:8, 1:3 and 1:0, using QDs at 0.25 μM. The experiment was performed in duplicate.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

P.E.D. acknowledges the National Institutes of Health R01NS087070. V.P. acknowledges Ramón Areces Foundation for their financial support. I.L.M. acknowledges DTRA and the NRL Nanosciences Institute for financial support. S.A.D. acknowledges an American Society of Engineering Education fellowship through NRL.

ABBREVIATIONS

QD	Quantum dot
LAP	leucine aminopeptidase
FRET	Förster resonance energy transfer
LA	lipoic acid

REFERENCES

1. Hofmann S, Lu JY. Molecular Basis of Infantile Batten Disease: Impact of Disordered Palmitoylated Protein Degradation on the Nervous System. FASEB J. 2014; 28:466.
2. Raorane DA, Lim MD, Chen FF, Craik CS, Majumdar A. Quantitative and Label-Free Technique for Measuring Protease Activity and Inhibition Using a Microfluidic Cantilever Array. Nano Lett. 2008; 8:2968–2974. [PubMed: 18720973]
3. Lopez-Otin C, Bond JS. Proteases: Multifunctional Enzymes in Life and Disease. J. Biol. Chem. 2008; 283:30433–30437. [PubMed: 18650443]
4. Schuliga M. The Inflammatory Actions of Coagulant and Fibrinolytic Proteases in Disease. Mediators Inflamm. 2015; 2015:437695. [PubMed: 25878399]
5. Safavi F, Rostami A. Role of Serine Proteases in Inflammation: Bowman-Birk Protease Inhibitor (BBI) as a Potential Therapy for Autoimmune Diseases. Exp. Mol. Pathol. 2012; 93:428–433. [PubMed: 23022357]
6. Cammer W, Bloom BR, Norton WT, Gordon S. Degradation of Basic Protein in Myelin by Neutral Proteases Secreted by Stimulated Macrophages: a Possible Mechanism of Inflammatory Demyelination. Proc. Natl. Acad. Sci. U. S. A. 1978; 75:1554–1558. [PubMed: 148651]
7. Poreba M, McGowan S, Skinner-Adams TS, Trenholme KR, Gardiner DL, Whisstock JC, To J, Salvesen GS, Dalton JP, Drag M. Fingerprinting the Substrate Specificity of M1 and M17 Aminopeptidases of Human Malaria, Plasmodium Falciparum. PLoS ONE. 2012; 7:e31938. [PubMed: 22359643]
8. Drag M, Bogyo M, Ellman JA, Salvesen GS. Aminopeptidase Fingerprints, an Integrated Approach for Identification of Good Substrates and Optimal Inhibitors. J. Biol. Chem. 2010; 285:3310–3318. [PubMed: 19948737]
9. Lin L, Sohar I, Lackland H, Lobel P. The Human CLN2 Protein/Tripeptidyl-Peptidase I Is a Serine Protease That Autoactivates at Acidic pH. J. Biol. Chem. 2001; 276:2249–2255. [PubMed: 11054422]

10. Dalal S, Ragheb DRT, Klemba M. Engagement of the S1, S1′ and S2′ Subsites Drives Efficient Catalysis of Peptide Bond Hydrolysis by the M1-Family Aminopeptidase from Plasmodium Falciparum. Mol. Biochem. Parasitol. 2012; 183:70–77. [PubMed: 22348949]
11. Carmel A, Yaron A. An Intramolecularly Quenched Fluorescent Tripeptide as a Fluorogenic Substrate of Angiotensin-I-Converting Enzyme and of Bacterial Dipeptidyl Carboxypeptidase. Eur. J. Biochem. 1978; 87:265–273. [PubMed: 208842]
12. Resch-Genger U, Grabolle M, Cavaliere-Jaricot S, Nitschke R, Nann T. Quantum Dots *versus* Organic Dyes as Fluorescent Labels. Nat. Methods. 2008; 5:763–775. [PubMed: 18756197]
13. Marmé N, Knemeyer J-P, Wolfrum J, Sauer M. Highly Sensitive Protease Assay Using Fluorescence Quenching of Peptide Probes Based on Photoinduced Electron Transfer. Angew. Chem. Int. Ed. 2004; 43:3798–3801.
14. Medintz IL, Stewart MH, Trammell SA, Susumu K, Delehanty JB, Mei BC, Melinger JS, Blanco-Canosa JB, Dawson PE, Mattoussi H. Quantum-Dot/Dopamine Bioconjugates Function as Redox Coupled Assemblies for *in Vitro* and Intracellular pH Sensing. Nat. Mater. 2010; 9:676–684. [PubMed: 20651808]
15. Klostranec JM, Chan WCW. Quantum Dots in Biological and Biomedical Research: Recent Progress and Present Challenges. Adv. Mater. 2006; 18:1953–1964.
16. Michalet X, Pinaud FF, Bentolila LA, Tsay JM, Doose S, Li JJ, Sundaresan G, Wu AM, Gambhir SS, Weiss S. Quantum Dots for Live Cells *in Vivo* Imaging, and Diagnostics. Science. 2005; 307:538–544. [PubMed: 15681376]
17. Barroso MM. Quantum Dots in Cell Biology. J. Histochem. Cytochem. 2011; 59:237–251. [PubMed: 21378278]
18. Alivisatos AP, Gu W, Larabell C. Quantum Dots as Cellular Probes. Annu. Rev. Biomed. Eng. 2005; 7:55–76. [PubMed: 16004566]
19. Mattoussi H, Palui G, Na HB. Luminescent Quantum Dots as Platforms for Probing *in Vitro* and *in Vivo* Biological Processes. Adv. Drug Deliv. Rev. 2012; 64:138–166. [PubMed: 21982955]
20. Ebrecht R, Don Paul C, Wouters FS. Fluorescence Lifetime Imaging Microscopy in the Medical Sciences. Protoplasma. 2014; 251:293–305. [PubMed: 24390249]
21. Holzinger M, Le Goff A, Cosnier S. Nanomaterials for Biosensing Applications: a Review. Front. Chem. 2014; 2:63. [PubMed: 25221775]
22. Knudsen BR, Jepsen ML, Ho YP. Quantum Dot-Based Nanosensors for Diagnosis *via* Enzyme Activity Measurement. Expert. Rev. Mol. Diagn. 2013; 13:367–375. [PubMed: 23638819]
23. Carregal-Romero S, Caballero-Diaz E, Beqa L, Abdelmonem AM, Ochs M, Huhn D, Suau BS, Valcarcel M, Parak WJ. Multiplexed Sensing and Imaging with Colloidal Nano- and Microparticles. Annu. Rev. Anal. Chem. (Palo Alto Calif). 2013; 6:53–81. [PubMed: 23451718]
24. Wegner KD, Hildebrandt N. Quantum Dots: Bright and Versatile *in Vitro* and *in Vivo* Fluorescence Imaging Biosensors. Chem. Soc. Rev. 2015; 44:4792–4834. [PubMed: 25777768]
25. Zhou M, Ghosh I. Quantum Dots and Peptides: a Bright Future Together. Biopolymers. 2007; 88:325–339. [PubMed: 17167795]
26. Clapp AR, Medintz IL, Mauro JM, Fisher BR, Bawendi MG, Mattoussi H. Fluorescence Resonance Energy Transfer Between Quantum Dot Donors and Dye-Labeled Protein Acceptors. J. Am. Chem. Soc. 2004; 126:301–310. [PubMed: 14709096]
27. Algar WR, Kim H, Medintz IL, Hildebrandt N. Emerging non-Traditional Förster Resonance Energy Transfer Configurations with Semiconductor Quantum Dots: Investigations and Applications. Coord. Chem. Rev. 2014; 263–264:65–85.
28. Medintz IL, Clapp AR, Brunel FM, Tiefenbrunn T, Tetsuo Uyeda H, Chang EL, Deschamps JR, Dawson PE, Mattoussi H. Proteolytic Activity Monitored by Fluorescence Resonance Energy Transfer Through Quantum-Dot-Peptide Conjugates. Nat. Mater. 2006; 5:581–589. [PubMed: 16799548]
29. Algar WR, Ancona MG, Malanoski AP, Susumu K, Medintz IL. Assembly of a Concentric Förster Resonance Energy Transfer Relay on a Quantum Dot Scaffold: Characterization and Application to Multiplexed Protease Sensing. ACS Nano. 2012; 6:11044–11058. [PubMed: 23215458]

30. Berti L, Medintz IL, Tom J, Mathies RA. Energy-Transfer Cassette Labeling for Capillary Array Electrophoresis Short Tandem Repeat DNA Fragment Sizing. Bioconjugate Chem. 2001; 12:493–500.
31. Huang S, Qiu H, Xiao Q, Huang C, Su W, Hu B. A Simple QD–FRET Bioprobe for Sensitive and Specific Detection of Hepatitis B Virus DNA. J. Fluoresc. 2013; 23:1089–1098. [PubMed: 23722996]
32. Qiu X, Hildebrandt N. Rapid and Multiplexed MicroRNA Diagnostic Assay Using Quantum Dot-Based Förster Resonance Energy Transfer. ACS Nano. 2015; 9:8449–8457. [PubMed: 26192765]
33. Claussen JC, Algar WR, Hildebrandt N, Susumu K, Ancona MG, Medintz IL. Biophotonic Logic Devices Based on Quantum Dots and Temporally-Staggered Forster Energy Transfer Relays. Nanoscale. 2013; 5:12156–12170. [PubMed: 24056977]
34. Boeneman K, Delehanty JB, Susumu K, Stewart MH, Deschamps JR, Medintz IL. Quantum Dots and Fluorescent Protein FRET-Based Biosensors. Adv. Exp. Med. Biol. 2012; 733:63–74. [PubMed: 22101713]
35. Chen N-T, Cheng S-H, Liu C-P, Souris J, Chen C-T, Mou C-Y, Lo L-W. Recent Advances in Nanoparticle-Based Förster Resonance Energy Transfer for Biosensing, Molecular Imaging and Drug Release Profiling. Int. J. Mol. Sci. 2012; 13:16598. [PubMed: 23443121]
36. Naqvi N, Liu K, Graham RM, Husain A. Molecular Basis of Exopeptidase Activity in the C-terminal Domain of Human Angiotensin I-Converting Enzyme: Insights into the Origins of its Exopeptidase Activity. J. Biol. Chem. 2005; 280:6669–6675. [PubMed: 15615692]
37. Aertgeerts K, Levin I, Shi L, Snell GP, Jennings A, Prasad GS, Zhang Y, Kraus ML, Salakian S, Sridhar V, et al. Structural and Kinetic Analysis of the Substrate Specificity of Human Fibroblast Activation Protein Alpha. J. Biol. Chem. 2005; 280:19441–19444. [PubMed: 15809306]
38. Kramer RA, Zandwijken D, Egmond MR, Dekker N. *In Vitro* Folding, Purification and Characterization of Escherichia Coli Outer Membrane Protease OmpT. Eur. J. Biochem. 2000; 267:885–893. [PubMed: 10651827]
39. Liu T, Han L-L, Du C-M, Yu Z-Y. Redox Potentials of Dopamine and its Supramolecular Complex with Aspartic Acid. Russ. J. Phys. Chem. B. 2014; 88:1085–1090.
40. Clarke SJ, Hollmann CA, Zhang ZJ, Suffern D, Bradforth SE, Dimitrijevic NM, Minarik WG, Nadeau JL. Photophysics of Dopamine-Modified Quantum Dots and Effects on Biological Systems. Nat. Mater. 2006; 5:409–417. [PubMed: 16617348]
41. Ji X, Palui G, Avellini T, Na HB, Yi C, Knappenberger KL Jr, Mattoussi H. On the pH-Dependent Quenching of Quantum Dot Photoluminescence by Redox Active Dopamine. J. Am. Chem. Soc. 2012; 134:6006–6017. [PubMed: 22394283]
42. Han L, Liu M, Ye D, Zhang N, Lim E, Lu J, Jiang C. Tumor Cell Membrane-Targeting pH-Dependent Electron Donor-Acceptor Fluorescence Systems with Low Background Signals. Biomaterials. 2014; 35:2952–2960. [PubMed: 24388814]
43. Wang G-L, Jiao H-J, Liu K-L, Wu X-M, Dong Y-M, Li Z-J, Zhang C. A Novel Strategy for the Construction of Photoelectrochemical Sensors Based on Quantum Dots and Electron Acceptor: The Case of Dopamine Detection. Electrochem. Commun. 2014; 41:47–50.
44. Gill R, Freeman R, Xu J-P, Willner I, Winograd S, Shweky I, Banin U. Probing Biocatalytic Transformations with CdSe−ZnS Quantum Dots. J. Am. Chem. Soc. 2006; 128:15376–15377. [PubMed: 17131995]
45. Burdine L, Gillette TG, Lin H-J, Kodadek T. Periodate-Triggered Cross-Linking of DOPA-Containing Peptide−Protein Complexes. J. Am. Chem. Soc. 2004; 126:11442–11443. [PubMed: 15366882]
46. Wilker JJ. Biomaterials: Redox and Adhesion on the Rocks. Nat. Chem. Biol. 2011; 7:579–580. [PubMed: 21849996]
47. Poreba M, Mihelic M, Krai P, Rajkovic J, Krezel A, Pawelczak M, Klemba M, Turk D, Turk B, Latajka R, et al. Unnatural Amino Acids Increase Activity and Specificity of Synthetic Substrates for Human and Malarial Cathepsin C. Amino Acids. 2014; 46:931–943. [PubMed: 24381006]
48. Stewart MH, Huston AL, Scott AM, Oh E, Algar WR, Deschamps JR, Susumu K, Jain V, Prasuhn DE, Blanco-Canosa J, et al. Competition between Förster Resonance Energy Transfer and Electron

Transfer in Stoichiometrically Assembled Semiconductor Quantum Dot–Fullerene Conjugates. ACS Nano. 2013; 7:9489–9505. [PubMed: 24128175]

49. Prasuhn DE, Blanco-Canosa JB, Vora GJ, Delehanty JB, Susumu K, Mei BC, Dawson PE, Medintz IL. Combining Chemoselective Ligation with Polyhistidine-driven Self-Assembly for the Modular Display of Biomolecules on Quantum Dots. ACS Nano. 2010; 4:267–278. [PubMed: 20099912]
50. Ji X, Wang W, Mattoussi H. Effects of Separation Distance on the Charge Transfer Interactions in Quantum Dot-Dopamine Assemblies. PCCP. 2015; 17:10108–10117. [PubMed: 25789996]
51. Blanco-Canosa JB, Medintz IL, Farrell D, Mattoussi H, Dawson PE. Rapid Covalent Ligation of Fluorescent Peptides to Water Solubilized Quantum Dots. J. Am. Chem. Soc. 2010; 132:10027–10033. [PubMed: 20597509]
52. Hu B-H, Messersmith PB. Protection of 3,4-Dihydroxyphenylalanine (DOPA) for Fmoc Solid-Phase Peptide Synthesis. Tetrahedron Lett. 2000; 41:5795–5798.
53. Prasuhn DE, Deschamps JR, Susumu K, Stewart MH, Boeneman K, Blanco-Canosa JB, Dawson PE, Medintz IL. Polyvalent Display and Packing of Peptides and Proteins on Semiconductor Quantum Dots: Predicted *versus* Experimental Results. Small. 2010; 6:555–564. [PubMed: 20077423]
54. Lee H, Scherer NF, Messersmith PB. Single-Molecule Mechanics of Mussel Adhesion. Proc. Natl. Acad. Sci. U. S. A. 2006; 103:12999–13003. [PubMed: 16920796]
55. Susumu K, Oh E, Delehanty JB, Blanco-Canosa JB, Johnson BJ, Jain V, Hervey WJ, Algar WR, Boeneman K, Dawson PE, et al. Multifunctional Compact Zwitterionic Ligands for Preparing Robust Biocompatible Semiconductor Quantum Dots and Gold Nanoparticles. J. Am. Chem. Soc. 2011; 133:9480–9496. [PubMed: 21612225]
56. Vulfson E, Ahmed G, Gill I, Goodenough P, Kozlov I, Law B. L-Dopa Ester Synthesis Catalyzed by α-Chymotrypsin in Organic Solvents. Biotechnol. Lett. 1990; 12:597–602.
57. Cohen GH, Silverton EW, Davies DR. Refined Crystal Structure of γ-Chymotrypsin at 1.9 Å Resolution. J. Mol. Biol. 1981; 148:449–479. [PubMed: 6914398]
58. Light, A. Methods Enzymol. Academic Press; 1967. Leucine aminopeptidase (LAP); p. 426-436.
59. Bradshaw, R.; Arfin, S. Aminopeptidases. Berlin Heidelberg: Springer; 1996. Methionine Aminopeptidase: Structure and Function; p. 91-106.
60. Venkatachalapathi YV, Balaram P. An Incipient 310 Helix in Piv-Pro-Pro-Ala-NHMe as a Model for Peptide Folding. Nature. 1979; 281:83–84. [PubMed: 551271]
61. Yamaguchi H, Kodama H, Osada S, Kato F, Jelokhani-Niaraki M, Kondo M. Effect of α,α-Dialkyl Amino Acids on the Protease Resistance of Peptides. Biosci. Biotechnol. Biochem. 2003; 67:2269–2272. [PubMed: 14586119]
62. Karle IL, Balaram P. Structural Characteristics of Alpha-Helical Peptide Molecules Containing Aib Residues. Biochemistry. 1990; 29:6747–6756. [PubMed: 2204420]
63. Algar WR, Malonoski A, Deschamps JR, Blanco-Canosa JB, Susumu K, Stewart MH, Johnson BJ, Dawson PE, Medintz IL. Proteolytic Activity at Quantum Dot-Conjugates: Kinetic Analysis Reveals Enhanced Enzyme Activity and Localized Interfacial “Hopping”. Nano Lett. 2012; 12:3793–3802. [PubMed: 22731798]
64. Díaz S, Malonoski A, Susumu K, Hofele R, Oh E, Medintz I. Probing the Kinetics of Quantum Dot-Based Proteolytic Sensors. Anal. Bioanal. Chem. 2015; 407:7307–7318. [PubMed: 26215169]
65. Beattie RE, Guthrie DJ, Elmore DT, Williams CH, Walker B. An Improved Spectrophotometric Assay for Leucine Aminopeptidase. Biochem. J. 1987; 242:281–283. [PubMed: 3593241]
66. Díaz, SA.; Breger, JC.; Medintz, IL. Chapter Two - Monitoring Enzymatic Proteolysis Using Either Enzyme- or Substrate-Bioconjugated Quantum Dots. In: Challa Vijaya, K., editor. Methods Enzymol. Academic Press; 2016. p. 19-54.
67. Schnolzer M, Alewood P, Jones A, Alewood D, Kent SB. *In Situ* Neutralization in Boc-Chemistry Solid Phase Peptide Synthesis. Rapid, High Yield Assembly of Difficult Sequences. Int. J. Pept. Protein Res. 1992; 40:180–193. [PubMed: 1478777]
68. Snee PT, Chan Y, Nocera DG, Bawendi MG. Whispering-Gallery-Mode Lasing from a Semiconductor Nanocrystal/Microsphere Resonator Composite. Adv. Mater. 2005; 17:1131–1136.
69. Susumu K, Oh E, Delehanty JB, Pinaud F, Gemmill KB, Walper S, Breger J, Schroeder MJ, Stewart MH, Jain V, Whitaker CM, et al. A New Family of Pyridine-Appended Multidentate

Polymers As Hydrophilic Surface Ligands for Preparing Stable Biocompatible Quantum Dots. Chem. Mater. 2014; 26:5327–5344.

70. Susumu K, Uyeda HT, Medintz IL, Pons T, Delehanty JB, Mattoussi H. Enhancing the Stability and Biological Functionalities of Quantum Dots *via* Compact Multifunctional Ligands. J. Am. Chem. Soc. 2007; 129:13987–13996. [PubMed: 17956097]

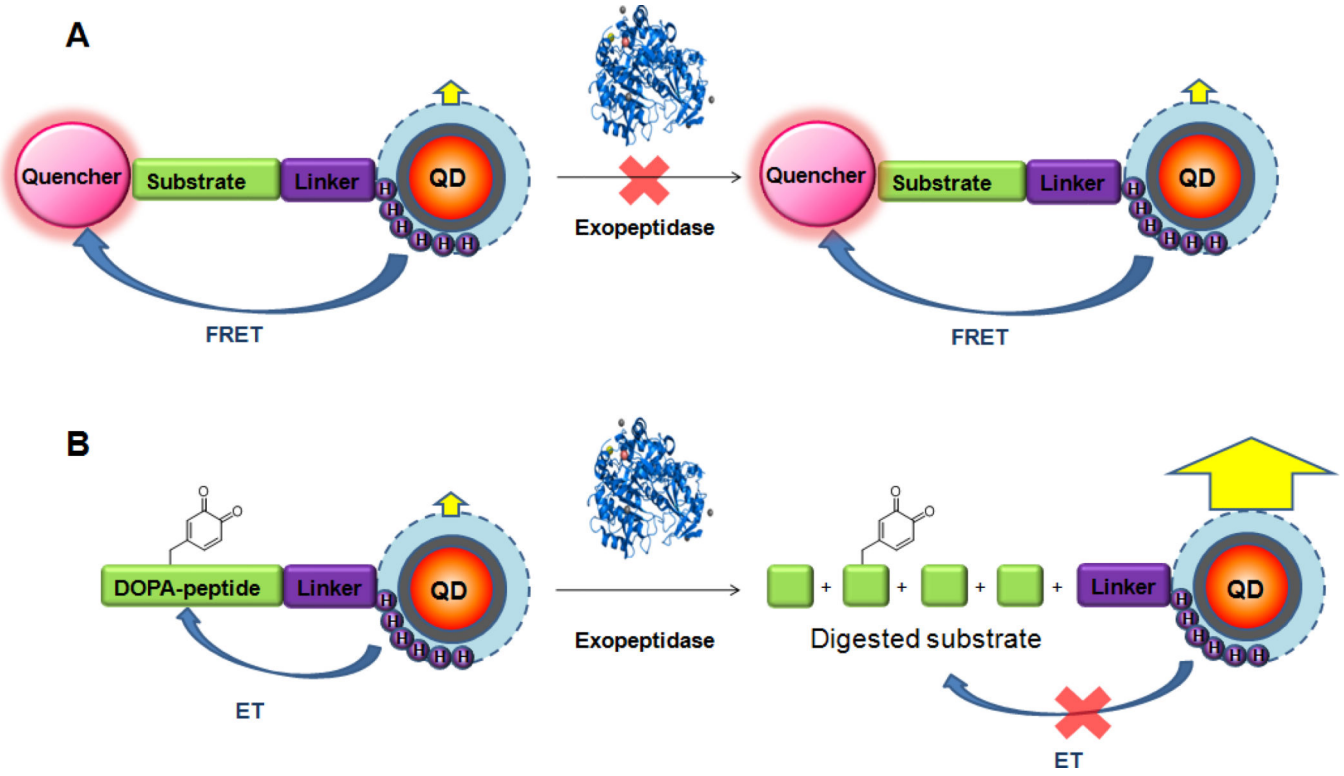


Figure 1.
FRET-based reporting is incompatible with exopeptidase monitoring (A). Strategy for exopeptidase activity sensing with QD/DOPA peptide bioconjugates (B).

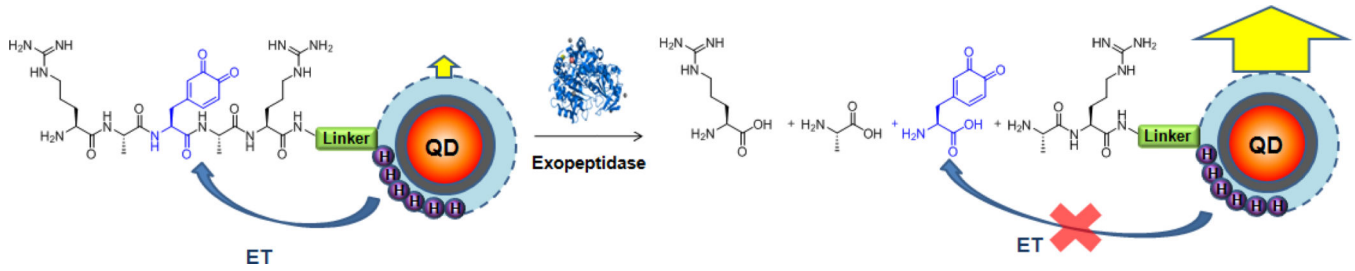


Figure 2.
Strategy for exopeptidase activity sensing with QD/DOPA peptide bioconjugates.

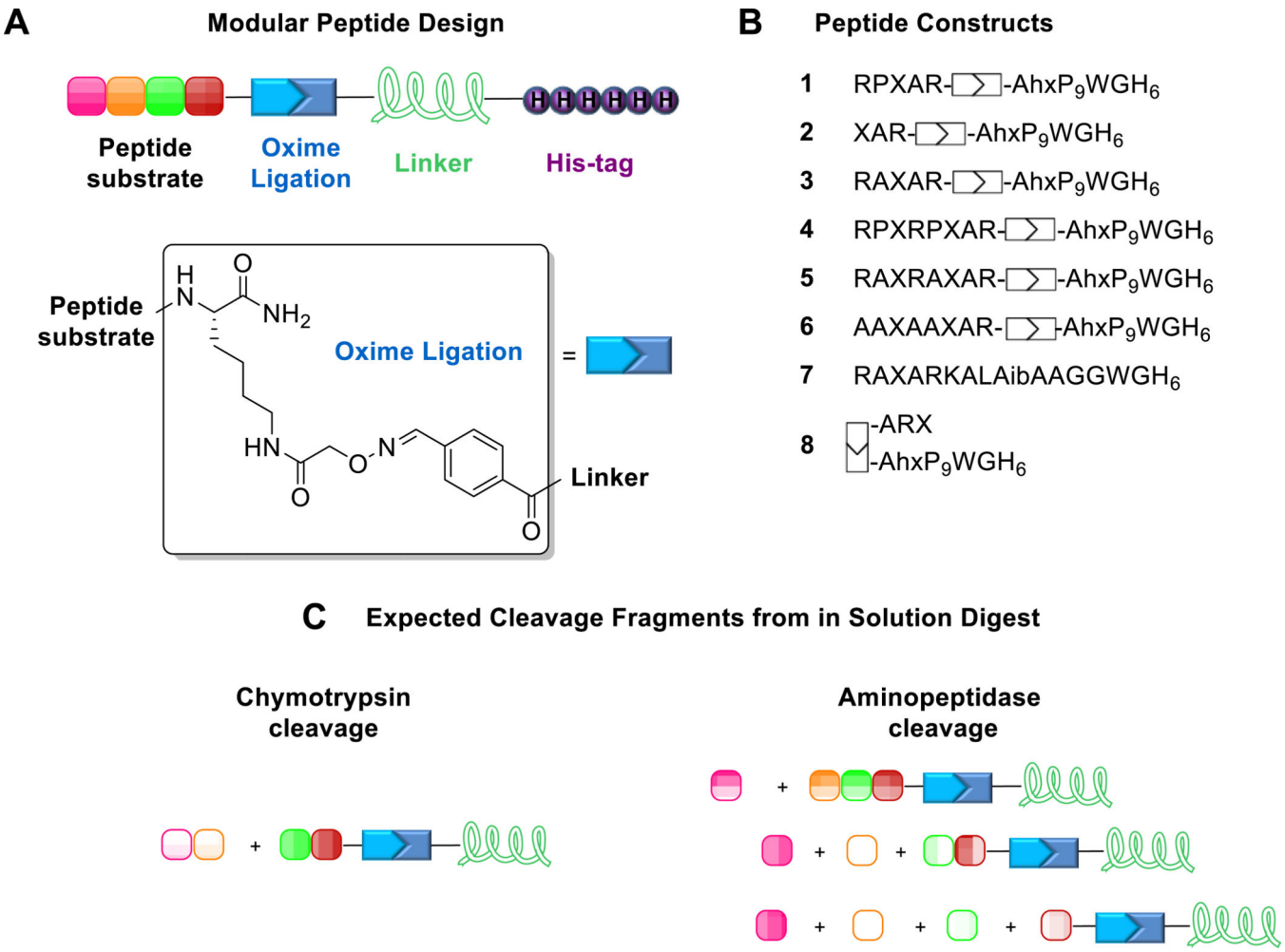


Figure 3.

Modular synthesis of DOPA-containing peptide substrates (A). Constructs synthesized (B) and expected enzyme digestion products of constructs (C). X=L-DOPA, Ahx=6-Aminohexanoic acid, Aib=2-Aminoisobutyric acid, all C-terminal amides.

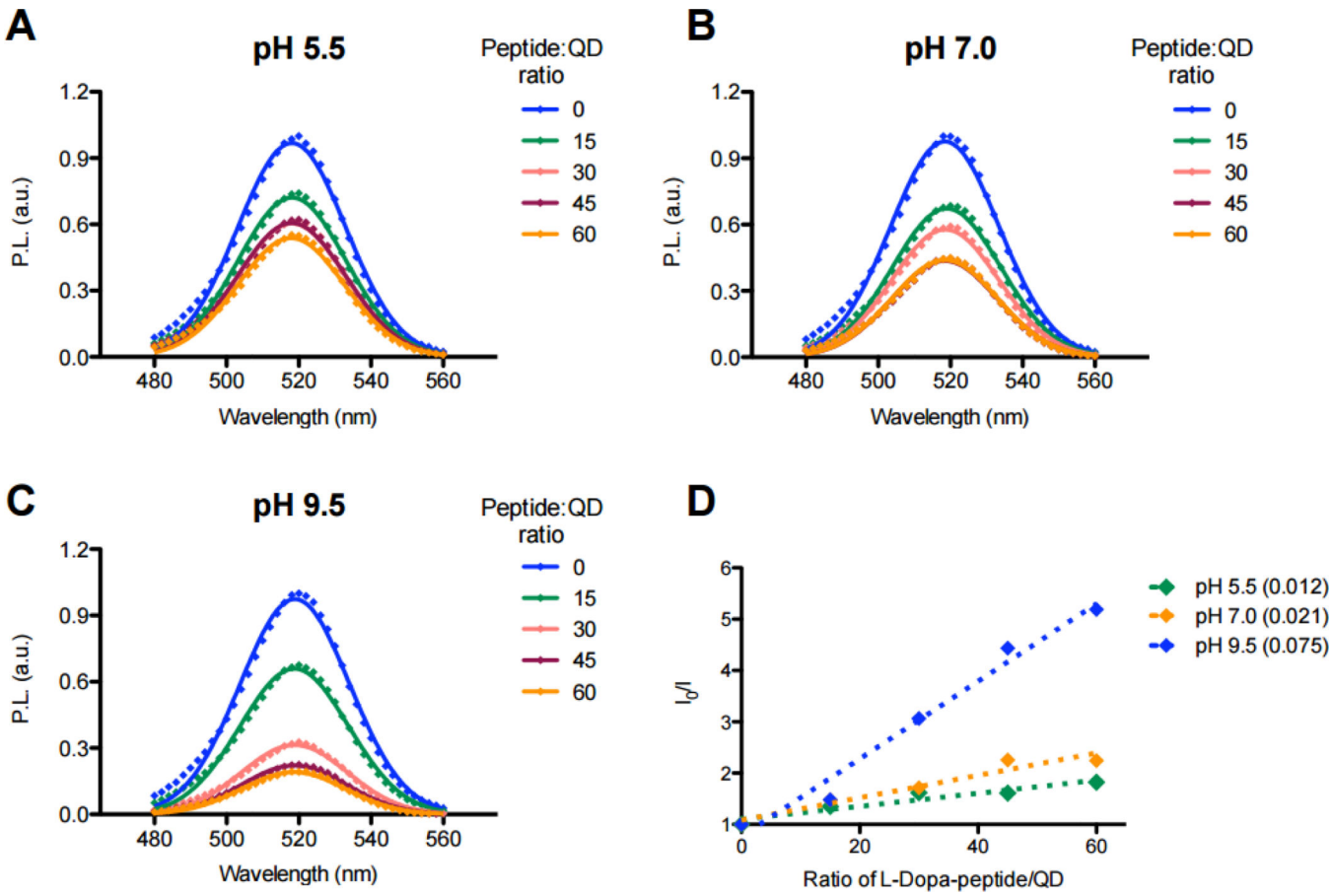


Figure 4.

QD photoluminescence is quenched by self-assembled DOPA containing peptides. Photoluminescence spectra collected from 523-nm-emitting DHLA-PEG-QDs self-assembled with an increasing ratio of peptide **3** added to PBS buffer at pH 5.5 (A), pH 7.0 (B) and 9.5 (C). Spectra were collected on an AVIV spectrofluorometer with 350 nm excitation. (D) Stern-Volmer plots of an increasing ratio of DOPA peptide per QD versus I_0/I performed at pH 5.5, 7.0 and 9.5 (data from A, B and C). Values in parenthesis are slopes derived for each data set reflecting the higher I_0/I for increasing pH values.

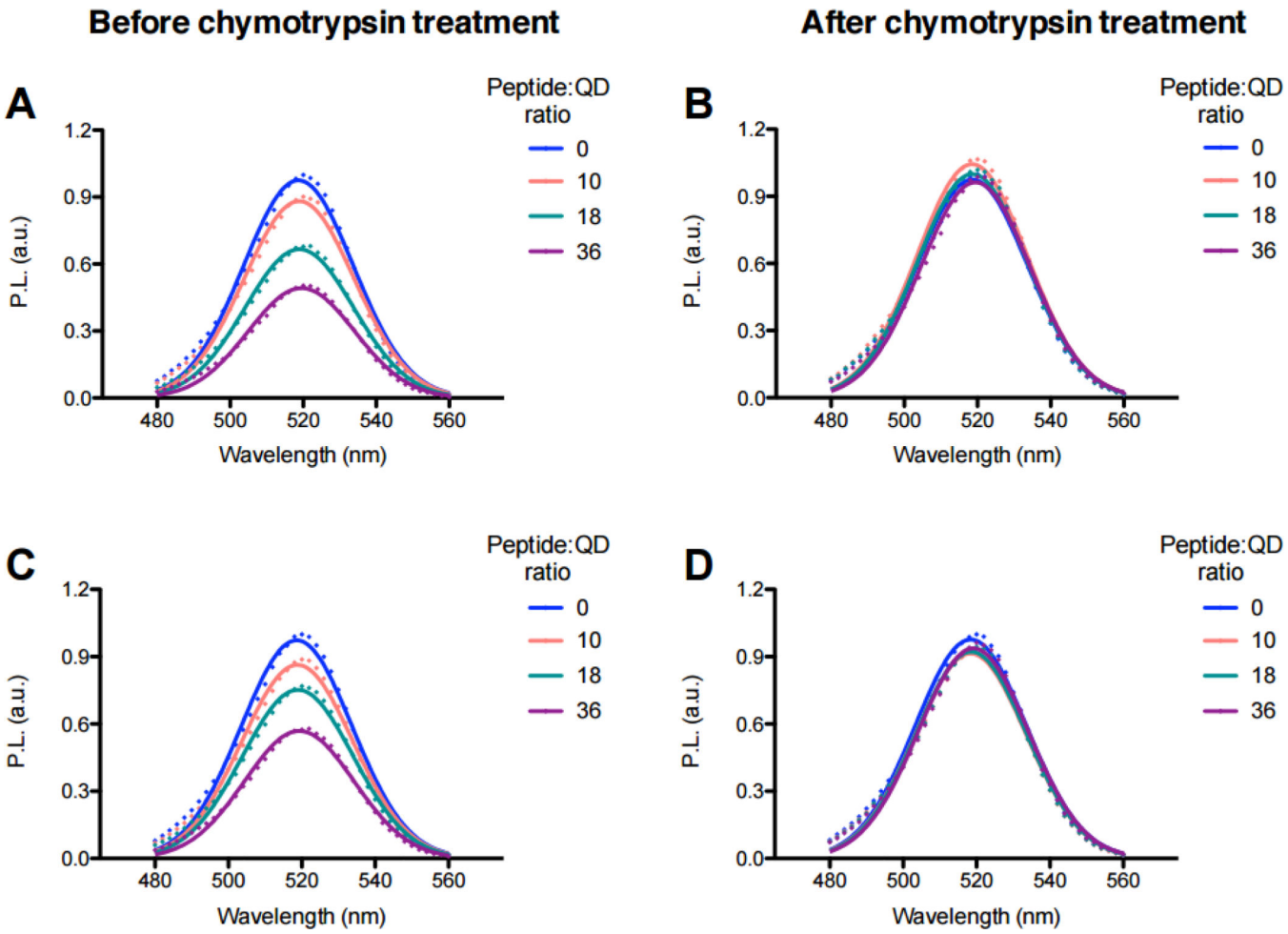


Figure 5.

Photoluminescence restored after chymotrypsin treatment. Normalized photoluminescence spectra of SQQDs at different peptide/DHLA-CL4 QD ratio before (A), (C) and after (B), (D) peptidase treatment, with peptides **1** (A), (B) and **3** (C), (D).

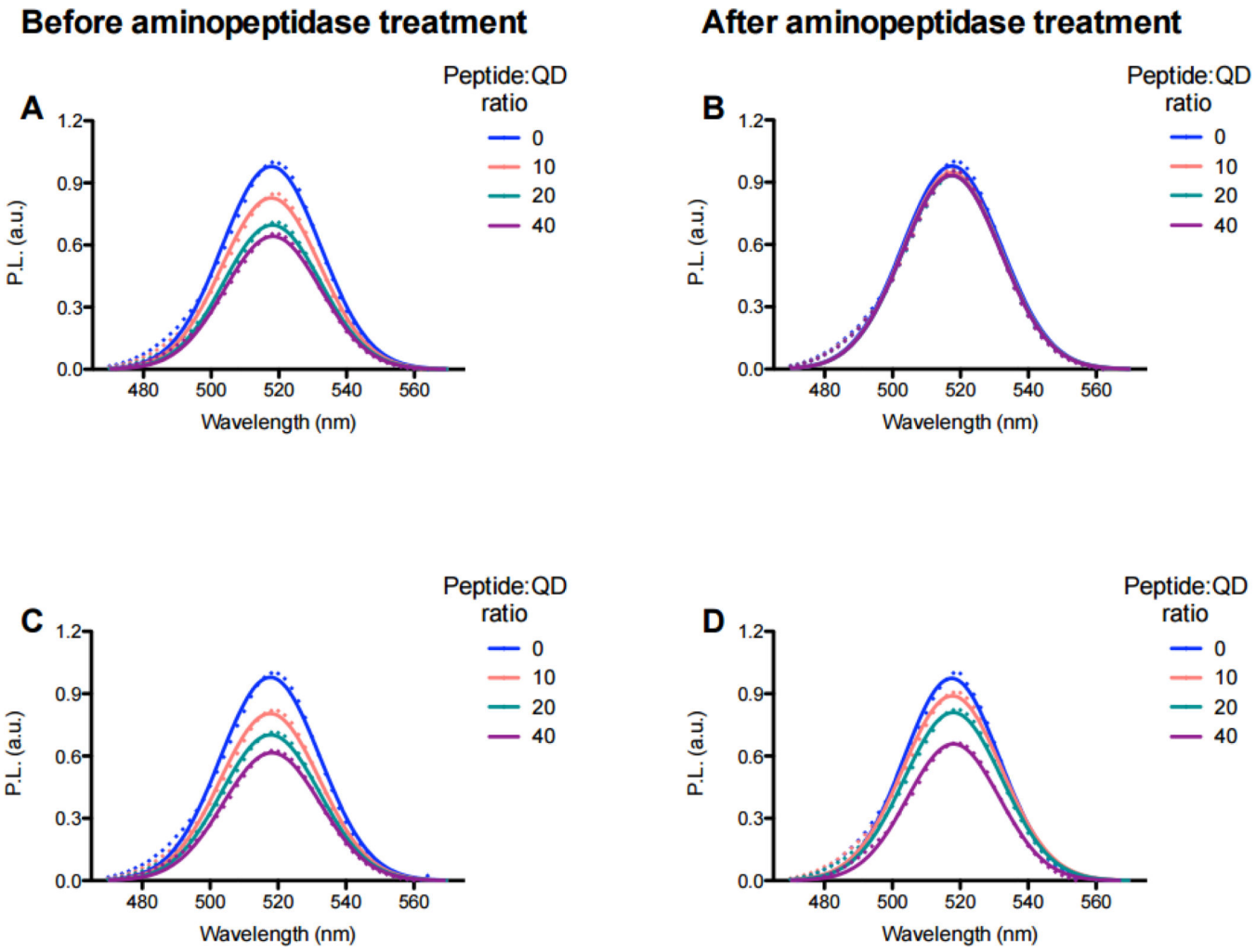


Figure 6.

Specificity of aminopeptidase activity reporting by SQQDs. (A), (B) Restoration of photoluminescence after exopeptidase treatment. Normalized photoluminescence spectra of DHLA-PEG-QD/DOPA bioconjugates with peptide **1** at 0, 10, 20 and 40 peptide/DHLA-PEG-QD ratio before (A) and after (B) aminopeptidase treatment. (C), (D) Lack of photoluminescence gain using a non cleavable substrate upon aminopeptidase treatment. Normalized photoluminescence spectra of DHLA-PEG-QD/DOPA bioconjugates with peptide **8** at 0, 10, 20 and 40 peptide/DHLA-PEG-QD ratio before (C) and after (D) aminopeptidase treatment.

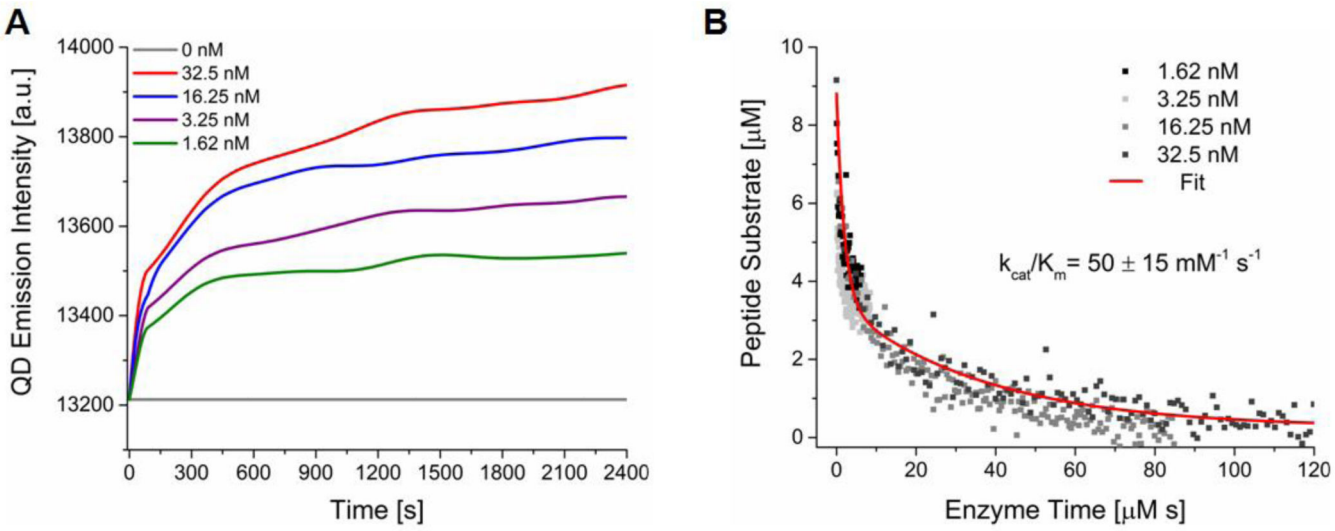


Figure 7.

Kinetic measurement of aminopeptidase QD-based enzyme sensor. A) Progress curves of QD emission. QD/DOPA peptide bioconjugates with peptide **2** at 15 peptide/DHLA-PEG-QD ratio, curves are varying enzyme concentration. Curves have been corrected for experimental drift observed in the plate reader.[66] B) Progress curve in enzyme time of aminopeptidase. The red curve corresponds to the integrated Michaelis-Menten fit to the data.