Supporting Information

An Approach to Zwitterionic Peptide Design for Colorimetric Detection of the Southampton Norovirus SV3CP Protease

Justin Yeung,[a] Zhicheng Jin,[b] Chuxuan Ling,[b] Maurice Retout,[b] Elany Barbosa da Silva,[c] Manan Damani,[c] Yu-Ci Chang,[d] Wonjun Yim,[d] Anthony J. O'Donoghue,[c] Jesse V. Jokerst[b,d,e]*

[a]Department of Bioengineering, University of California San Diego, La Jolla, CA 92093, United States

[b]Department of NanoEngineering, University of California, San Diego, La Jolla, CA 92093, United States

[c]Skaggs School of Pharmacy and Pharmaceutical Sciences, University of California, San Diego, La Jolla, CA 92093, United States

[d]Materials Science and Engineering Program, University of California, San Diego, La Jolla, CA 92093, United States

[e]Department of Radiology, University of California, San Diego, La Jolla, CA 92093, United States

***Corresponding author's email: jjokerst@ucsd.edu (J.V.J.)**

1. Materials

Sodium citrate tribasic dihydrate (>99%), gold(III) chloride trihydrate (HAuCl4 .3H2O, >99.9%), sodium dodecyl sulfate (SDS, >99%), Trizma® base (>99.9%), Trizma® hydrochloride (>90%), bis(p-sulfonatophenyl)phenylphosphine dihydrate dipotassium salt (BSPP, 97%), DL-dithiothreitol (DTT, >99%), trifluoroacetic acid (TFA, HPLC grade, >99%), 2,2'-(ethylenedioxy)diethanethiol (EDDET, 95%), and piperidine (ReagentPlus®, 99%) were purchased from Sigma Aldrich (St Louis, MO). Thioanisole (>99%), N,N-diisopropylethylamine (DIPEA, >99%), and triisopropylsilane (TIPS, >98%) were purchased from Tokyo Chemical Industry Co., Ltd. (TCI). Fmoc-protected amino acids, hexafluorophosphate benzotriazole tetramethyl uronium (HBTU), and Fmoc-rink amide MBHA resin (0.67 mmol/g, 100-150 mesh) were purchased from AAPPTec, LLC (Louisville, KY). The recombinant Southampton norovirus 3C-like protease (SV3CP) was expressed using the SV3CP plasmid in a previous report and purified accordingly.[1] The purified SV3CP was stored at −80 °C in 0.02M Tris-HCl, pH 8.0, 150 mM NaCl, 5 mM DTT, 5% glycerol. TBE buffer (10×, molecular biology certified) was from IBI Scientific, Inc (Dubuque, IA). A 10-mL disposable reaction vessel and the pressure caps for peptide-resin cleavage were purchased from Torviq Inc. (Tucson, AZ). Organic solvents including N,N-dimethylformamide (DMF, sequencing grade), acetonitrile (ACN, HPLC grade), ethyl ether (certified ACS), methylene chloride (DCM, certified ACS), and dimethyl sulfoxide (DMSO, certified ACS) were also from Fisher Scientific International, Inc. (Hampton, NH). Ultrapure water (18 MΩ.cm) was obtained from a Milli-Q Academic water purification system (Millipore S4 Corp., Billerica, MA). TEM grids (formvar/carbon 300 mesh Cu) were purchased from Ted Pella (Redding, CA). Pooled human saliva and single donor human fecal matter were purchased from Innovative Research (Novi, MI). The Amicon™ Ultra-15 centrifugal filter units (Mw cutoff = 50 kDa) were from MilliporeSigma (St. Louis, MO). Glassware and stir bars were cleaned with aqua regia (HCl:HNO3=3:1 by volume) and boiling DI water before use.

2. Materials Synthesis

2.1 Gold nanoparticle synthesis and characterization

Citrate-capped AuNPs (~13nm diameter, TEM) were first prepared using the Turkevich method through rapid injections of an aqueous solution of sodium citrate tribasic dihydrate (150 mg, 5 mL) into an aqueous solution of $HAuCl_4 \bullet 3H_2O$ (45 mg, 300 mL) under boiling conditions and vigorous stirring. The reaction was left to boil while stirring for an additional 20 minutes and was cooled to room temperature. The deep red dispersion was then purified by centrifuging the sample at 18,000 *g* for 40 minutes, discarding the supernatant. The resulting pellet of citrate-AuNPs was redispersed in DI water ($\varepsilon_{520}=4.0\times10^8$ $M^{-1}cm^{-1}$). BSPP-AuNPs were then prepared through a ligand exchange method. The citrate-AuNP dispersion (100 mL, 3.4 nM) was vigorously stirred overnight with BSPP (100 mg, 2 mL) in a round bottom flask. The mixture was then purified using syringe filtration (hydrophilic PTFE, 0.45 mm) followed by centrifugation and redispersed in DI water, following the citrate-AuNP procedure, with sonication for 10 minutes. BSPP-AuNPs were stored at 4°C for long-term use.

Size and zeta potential measurements were carried out to characterize the citrate- and BSPP-AuNPs. AuNPs were prepared in a 1 in 10 dilution and loaded in a dynamic light scattering (DLS) cuvette. The hydrodynamic size and zeta potential was measured in a Zetasizer Nano ZS90 (Malvern Panalytical, Inc.). Fourier transform infrared (FTIR) data was taken to confirm the presence of the ligand molecule on the particle surface, and was collected using a Bruker Tensor II FTIR spectrophotometer by drying the corresponding solution on the optical window. Transmission electron microscopy (TEM) was carried out by using 4 µL of particle solution dropped cast onto a plasma pre-cleaned carbon coated copper grid (300 mesh, Ted Pella). After drying, the grid was stained with 10 µL of 2% (w/v) uranyl acetate for 2.5 min and blotted on a Kimwipe. The staining process was repeated once as above. Then 10 µL of DI water was applied to wash the salt residues and the grid was dried at 37°C for TEM measurement (a JEOL 1200 EX II operated at 80 kV).

2.2 Peptide Synthesis

Peptides were synthesized using an automated Eclipse™ peptide synthesizer (AAPPTec, Louisville, KY) through standard solid phase Fmoc synthesis on Rink-amide resin. Peptides were chain assembled by Fmoc-SPPS (solid-phase peptide synthesis) on rink-amide-MBHA-resin (0.67 mmol/g, 200 mg). Amino acid coupling was performed under the protection of nitrogen gas with 0.2 M Fmoc-amino acid (5 equiv.) in 3 mL DMF, 0.2 M HBTU in DMF (5 equiv.) in 3 mL DMF, 0.3 M DIPEA (7.5 equiv.) in 3 mL DMF, and 20% (v/v) piperidine in 2×4 mL DMF for each cycle. The sequence analysis software tool (AAPTec) determined the number of coupling cycles. The resulting peptides on the resin were transferred into a syringe filter (Torviq Inc,), and washed with fives rounds of DCM (2 mL) each, to remove excess DMF, which were then dried under a vacuum. Peptide cleavage from the resin was done using a cleavage cocktail (3 mL) containing TFA (82.5%), EDDET (2.5%), phenol (5%), thioanisole (5%), and H_2O (5%). The peptide-resin cleavage mixture was incubated for 3 hours with gentle shaking. After cleavage, the resin was filtered, and the filtrate containing the crude peptide was precipitated and washed with three rounds of cold ether (15 mL, -20°C) and then suspended in 50% ACN/H2O (10 mL, v/v) to be lyophilized in a FreeZone Plus 2.5 freeze dry system (Labconco S5 Corp., Kansas, MO).

Peptide purification used a Shimadzu LC-40 HPLC system equipped with a LC-40D solvent delivery module, a photodiode array detector SPD-M40, and DGU-403 degassing unit. The crude sample was dissolved in an acetonitrile/H_2O mixture (1:1, v/v) and injected with a volume of 2 mL on a Zorbax 300 BS, C18 column (5 mm, 9.4×250 mm) from Agilent (Santa Clara, CA), and eluted at a flow rate of 1.5 mL/min

over a 40 min linear gradient from 10% to 95% of acetonitrile in water (with 0.05% TFA, HPLC grade). Fractions containing the pure peptide were confirmed by matrix-assisted laser desorption ionization–time-of-flight mass spectrometry (MALDI-TOF MS) (positive ion mode) using the Bruker Autoflex Max MALDI-TOFMS instrument in the Molecular MS Facility (MMSF) at UC San Diego. MALDI-TOF samples were prepared in an alpha-cyano-4-hydroxycinnamic acid (HCCA)/H_2O mixture (1:1, v/v). Desired fractions were then lyophilized in a FreeZone Plus 2.5 freeze dry system (Labconco S5 Corp., Kansas, MO).

Peptide concentrations were determined using a NanoDrop™ One UV-vis spectrophotometer (Thermo Fisher Scientific, Waltham, MA): The 31 method was applied for peptide quantification.[2] An absorption coefficient of ε_{205} = 31 mL•mg^{-1} cm^{-1} was used.

3. Norovirus SV3CP Protease Expression

The norovirus 3CP protease was expressed in *Escherichia coli* BL21(D3) cells from a pSV3C plasmid derived from pT7-7 as described previously.[1] A 20 mL starter E. coli culture in Luria-Bertani (LB) media containing carbenicillin (50 µg/mL) was grown ~15 h at 37°C with shaking. 10 mL of starter culture was used to inoculate 1 L of LB/carbenicillin media and incubated at 180 rpm until the OD reached 0.5 (~4 h). SV3CP expression was induced using 1 mM isopropyl β-d-1-thiogalactopyranoside (IPTG) at 37°C and 200 rpm. After 3 h, cells were collected by centrifugation at 12,000 rpm for 20 min at 4°C. The cell pellet was suspended in 50 mL of Buffer A consisting of 10 mM sodium phosphate buffer, pH 7.65 and 5 mM dithiothreitol (DTT). Approximately 2 mg of DNase I was added and cells were lysed by sonication on ice (2 sec ON, 5 sec OFF, 50% amplitude) for 5 cycles. The lysate was clarified by centrifugation at 12,000 rpm for 20 min at 4°C.

4. Norovirus SV3CP Protease Purification

Recombinant SV3CP was enriched from the supernatant using HiTrapCM FF (Cytiva) followed by HiTrapSP FF (Cytiva). All purification steps were carried out at 4°C and protein was clarified by passing through a 0.22-µm filter prior to loading on each column. Protein was loaded in Buffer A and eluted in linear gradient of Buffer B that consisted of 10 mM Phosphate buffer pH 7.65, 5 mM DTT and 1 M NaCl. Fractions were analyzed for purity by denaturing SDS-PAGE using a 4-12% Bis-Tris gel with MOPS running buffer and stained with Coomassie Blue. Fractions were also analyzed by a fluorescence-based activity assay using the peptide substrate, Ac-EFQLQ-7-amino-4-methylcoumarin that was custom synthesized by GenScript. Activity was measured in 384-well black plates on a Biotek Synergy HTX (excitiation 360 nm, emission 460 nm) at room temperature using 50 µM of fluorogenic substrate in 100 mM Tris pH 8.5 and 5 mM DTT. Fractions containing the highest enzyme activity were pooled and then buffer exchanged and concentrated using Buffer A in an Amicon Ultra centrifugal filter units with 3 kDa molecular weight cutoff (Millipore). The last step of purification was performed using the size-exclusion FPLC on the Superose 6 Increase 10/300 GL (GE Healthcare) column using Buffer A with 100 mM NaCl added. Fractions were analyzed by SDS-PAGE and by a fluorescence-based activity assay. Fractions containing the most amount of active enzyme were pooled and stored at -80°C.

5. Dynamic Range Measurements

The stock of each peptide substrate was prepared by dissolving the intact peptide in Tris-buffer (TB) (0.02M, pH 8.0, with 150 mM NaCl and 5 mM DTT). A solution of peptide fragments was also prepared by incubating the intact peptide solution with SV3CP at an enzyme:substrate molar ratio of 1:200 at 37°C for at least 48 hours to ensure maximal cleavage. After incubation, intact/fragment peptide stocks of the

desired final concentration (*e.g.*, 0.1, 0.2, 0.5, 1, 2, 5, 10, 20, 50, 100 μM) were injected into a 96-well plate. TB buffer and BSPP-AuNPs (100 μL, 3.4 nM) were added to reach a final volume of 120 μL. At least three replicates of each peptide/fragment were measured. Absorbance at 600 nm and 520 nm at room temperature was measured every 1 minute for 1 hour giving ratiometric signal (Abs_{600}/Abs_{520}). TB buffer was used as the blank. The 10-minute mark was extracted for analysis and quantification of the aggregation state to determine the critical coagulation concentration (CCC). The CCC was calculated using a statistical method previous reported in literature.[3] This study uses the value of the limit of detection (LoD) to give a close approximation of the CCC:

$$CCC \equiv LoD_{int.} = mean_{blank} + 1.645 \times (SD_{blank}) + 1.645 \times (SD_{low\ conc.\ sample})$$

(S1)

6. LoD measurement

The N6 peptide and BSPP-AuNPs were used to detect SV3CP. The SV3CP of the desired concentration (*e.g.*, 0, 1, 2, 5, 10, 20, 50, 100, 200, 500 nM) was mixed with the intact N6 peptide (c_{final} = 50 μM) in TB buffer and incubated at 36°C for 48 hours. At least three replicates of each experiment were made. After incubation, the assay was transferred to a 96-well plate and BSPP-AuNPs was added (100 μL, 3.4 nM) to achieve a final volume of 120 μL. The absorbance of the mixture at 600 and 520 nm were recorded in a microplate reader at room temperature every 1 min for 1 hour, and the ratiometric signal (Abs_{600}/Abs_{520}) at 10 minutes was extracted to determine the detection limit.

7. Enzyme-substrate Characterization

7.1 Enzyme kinetics for fluorogenic substrate

The kinetic model of an enzyme is largely developed by Michaelis and Menten[4] and Henri[5] with significant improvements by Briggs-Haldane[6]. Simply, the enzyme and substrate reversibly form an enzyme-substrate complex followed by dissociation of the intermediate complex to produce the product with free enzyme given as:

$$E + S \underset{k_{off}}{\overset{k_{on}}{\rightleftharpoons}} ES \underset{k_{-cat}}{\overset{k_{cat}}{\rightleftharpoons}} E + P$$

(S2)

Here, k_{on}, k_{off}, k_{cat}, and k_{-cat} are rate constants. The relationship between the initial velocity of product formation (υ) and the substrate concentration ($[S]_0$) is known as the classical Michaelis-Menten equation:

$$\upsilon = \frac{\upsilon_{max}}{K_M + [S]_0}$$

(S3)

where υ_{max} is the maximum velocity and K_M is the Michaelis-Menten constant. The subscript *0* denotes the total concentration. The initial velocity reaches ½ υ_{max} when $[S]_0 = K_M$,. Since the k_{cat} is the rate limiting step in enzymatic reactions, then:

$$\upsilon \equiv \frac{d[P]}{dt} = k_{cat}[ES] - k_{-cat}[E][P]$$

(S4)

Dissociation of the ES complex is usually considered as an irreversible process, due to the low affinity of enzyme to product, thus k_{-cat} is neglibile:

$$v \approx k_{cat}[ES] \; or \; v_{max} = k_{cat}[E]_0$$

(S5)

Experimentally, in a 384-well plate the fluorogenic substrate was diluted in TB buffer to reach a final $[S]_0$ of 0.1, 0.5, 1, 5, 10, 50, 100, 200, and 500 µM (*e.g.*, with respect to a 30 µL final volume). SV3CP ($[E]_0$ = 850 nM) was then added to each and the total volume was brought to 30 µL using TB buffer. The plate was then incubated at 37°C in a hybrid multi-mode microplate reader with 3 seconds of shaking before each cycle of reading and the photoluminescence intensity (excitation and emission at 360 and 460 nm respectively) was recorded over 5 hours with 2-minute intervals between each cycle. Measurements were done in triplicates. PL values at 30 minutes and 4 hours were averaged and recorded as PL_{30min} and PL_{max}, respectively. The PL_{max} was used to convert the PL_{30min} to initial velocity, V_0, which was then correlated to substrate concentration. Data was then fitted to the above Michaelis-Menten equation.

7.2 Specificity Test

Desired amounts of trypsin, hemoglobin, α-amylase, thrombin, and bovine serum albumin (BSA) equivalent to 100 nM in a 120 µL solution were spiked into TB buffer. The intact N6 peptide (50 µM) was added to the above mixtures in microtubes and incubated at 37°C for 48 hours. The mixtures were then transferred to a 96-well plate and BSPP-AuNPs were added (100 µL, 3.4 nM) to reach a final volume of 120 µL. The experiment was performed in triplicates, and the absorbance of the mixtures were recorded at 600 and 520 nm to obtain the ratiometric signal (Abs_{600}/Abs_{520}). The 10-minute mark was extracted for analyses.

8. Probing Interparticle Interactions

A dispersion of BSPP-AuNPs (100 µL, 3.4 nM) in a microtube was thoroughly mixed with the intact N8 peptide (1 µL, 1.55 mM) to induce aggregation until no further color change was observed. The mixture was then lightly centrifuged at 500 *g* for 1 minute discarding the supernatant afterwards. The probing solution (100 µL) was then added to the pellet and sonicated for 10 seconds. These solutions include: DI water, PEG_{2000} (10 mM), Triton X-100 (10 mM), HCl (10 mM), NaOH (10 mM, pH 11.0), urea (1 M), SDS (10 mM), DMSO (100%), DMF (100%), and an aqueous solution of BSPP (10 mM). The mixtures were transferred to a 96-well plate and the ratiometric signal (Abs_{600}/Abs_{520}) was recorded immediately. The positive and negative controls were BSPP-AuNP dispersions with N8 peptide and only BSPP-AuNP respectively.

9. Matrix Test

The system's ability to detect SV3CP in different biological matrices including external breath condensate (EBC), fecal matter, urine, 1% BSA, saliva, human plasma, and Dulbecco's modified Eagle's medium (DMEM) was tested. SV3CP of desired amount (equivalent to 100 nM in 120 µL) were spiked into the above matrices and incubated with intact N6 peptide at 37°C for 48 hours in microtubes. The mixtures were transferred into a 96-well plate and BSPP-AuNPs (100 µL, 3.4 nM) were added to reach a final volume of 120 µL. The experiment was performed in triplicates, and the absorbance of the mixtures were

recorded at 600 and 520 nm to obtain the ratiometric signal (Abs_{600}/Abs_{520}). The 10-minute mark was extracted for analyses.

10. References

1. R. J. Hussey, L. Coates, R. S. Gill, P. T. Erskine, S. F. Coker, E. Mitchell, J. B. Cooper, S. Wood, R. Broadbridge, I. N. Clarke, P. R. Lambden and P. M. Shoolingin-Jordan, *Biochemistry*, 2011, **50**, 240-249.
2. N. J. Anthis and G. M. Clore, *Protein Sci*, 2013, **22**, 851-858.
3. D. A. Armbruster and T. Pry, *Clin Biochem Rev*, 2008, **29 Suppl 1**, S49-52.
4. L. Michaelis, M. L. Menten, K. A. Johnson and R. S. Goody, *Biochemistry*, 2011, **50**, 8264-8269.
5. V. Henri, *Archivio di Fisiologia* 1904, **1**, 299-324.
6. G. E. Briggs and J. B. Haldane, *Biochem J*, 1925, **19**, 338-339.

Figures (Supporting Information)

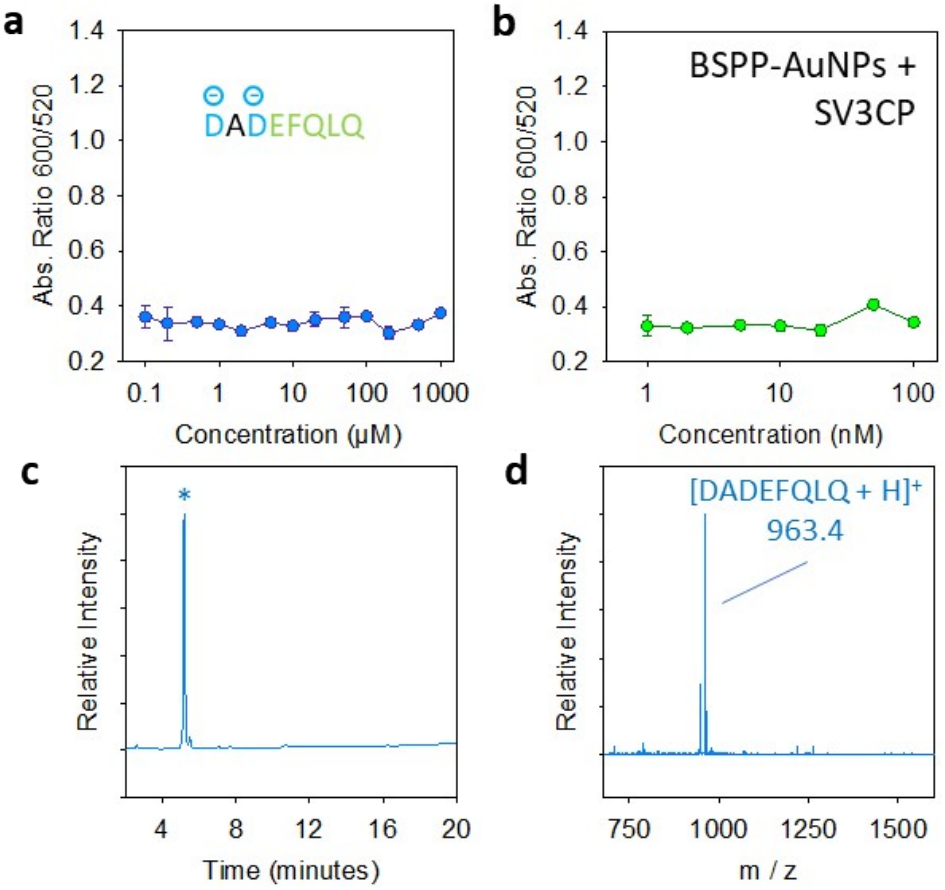

Figure S1. Aggregation control experiments using synthesized N-fragment and SV3CP. (a) Ratiometric absorbance as a function of synthesized N-fragment (DADEFQLQ). No aggregation of the BSPP-AuNPs was observed, even at concentration as high as 1000 μM. **(b)** Ratiometric absorbance as a function of SV3CP. The protease showed no aggregation of the particles, showing that the system cannot be activated by the protease alone. **(c)** HPLC and **(d)** MALDI-TOF MS confirm the purity of the synthesized N-fragment.

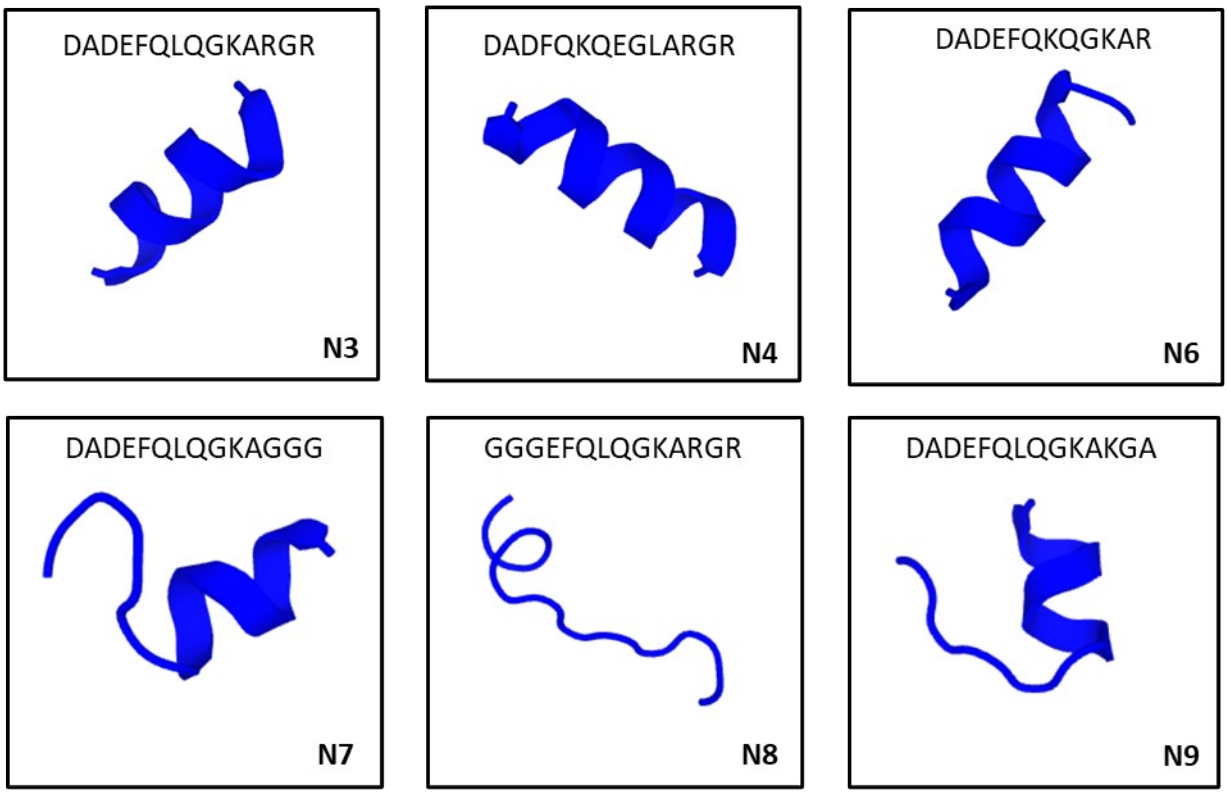

Figure S2. 3-D Computational folding images of peptide derivatives. Simulations of peptide folding were done using the publicly available PEP-FOLD 3 platform. The highest probable model for each peptide was reported. Observed structures include α-helixes, straight chains, and combinations thereof.

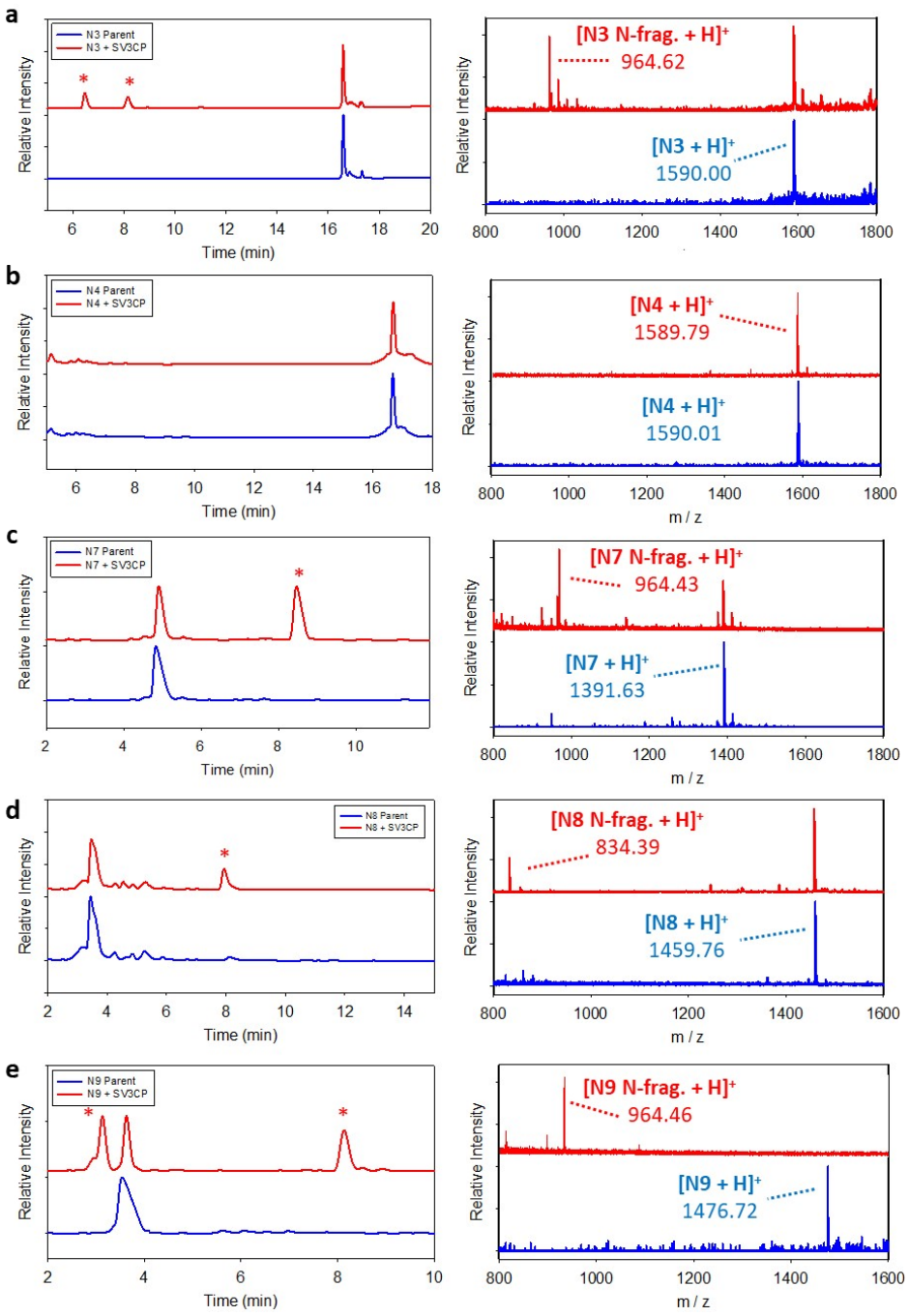

Figure S3. Confirmation of SV3CP-mediated cleavage of peptide derivatives. (left) HPLC before and after incubation with SV3CP and (right) MALDI-TOF MS before (blue) and after (red) incubation with SV3CP for pepeitdes **(a)** N3, (**b)** N4, **(c)** N7, **(d)** N8, and **(e)** N9. '*' denotes the presence of new peaks due to proteolysis. All peptides were incubated with SV3CP at a [E]:[S] ratio of 1:200 for 48 hours. Proteolysis is confirmed through the detection of the N-fragment. Note, MALDI-TOF MS analysis of the cleavage was conducted on the crude proteolysis reaction; mass spectrometry of individual HPLC peaks was not suitable due to the fractions having low yields.

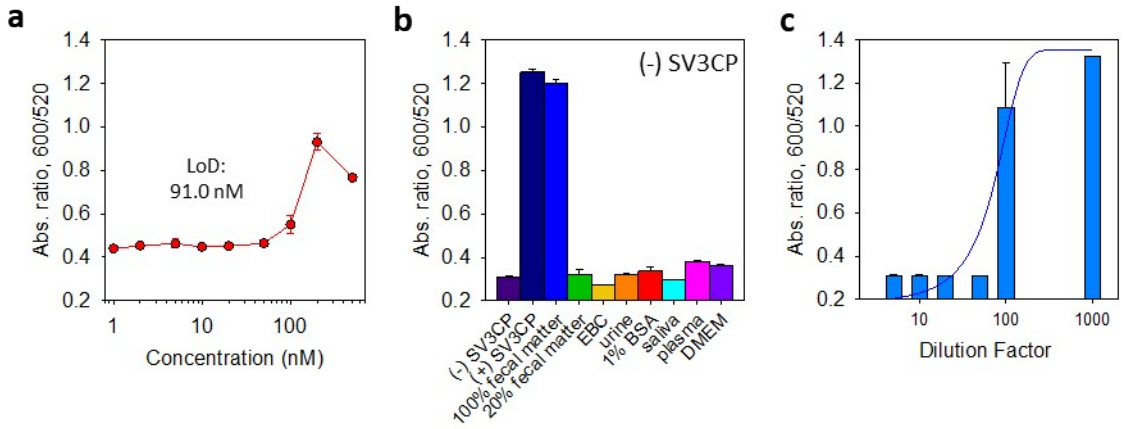

Figure S4. Limit of detection of SV3CP after 8 hours, nanoparticle colloidal stability in biological matrices, and fecal matter dilutions. (a) Ratiometric absorbance as a function of SV3CP concentration. N6 substrate (50 µM) and BSPP-AuNPs (3.4 nM) were employed. The substrate was incubated with the protease in TB buffer for 8 hours before addition of BSPP-AuNPs. **(b)** Ratiometric absorbance of BSPP-AuNPs (3.4 nM, 100 µL) in various biological matrices (20 µL). Particles were colloidally stable in all matrices except fecal matter, which required a minimum 1 in 5 dilution to prevent aggregation. (-) and (+) SV3CP in TB buffer were included as negative and positive controls respectively. **(c)** Ratiometric absorbance as a function of dilutions of fecal matter matrix. Fecal matter matrix was prepared by vortexing 100 mg of human feces in 1.5 mL of TB buffer for four minutes and centrifuging the resulting mixture at 20 x G for 15 seconds. The supernatant was collected and passed through a 50-kDa filter to produce the final product. A minimum dilution factor of 5 is needed to prevent matrix-induced aggregation of the AuNPs. Here, 100 nM of SV3CP was then incubated with the N6 substrate in varying dilution factors of fecal matter matrix (e.g., 5 – 1000) in 20 µL for 48 hours, followed by addition of BSPP-AuNPs (3.4 nM, 100 µL) for colorimetric readout.