Electronic Supplementary Material (ESI) for Chemical Communications.

Supplementary Materials for:

Localized and Regulated Peptide Pigment Formation Inside Liquid Droplets Through Confined Enzymatic Oxidation

Kenny Barriales, [a-c] Salma Kassem, [a] Deborah Semanta,[a] Alfredo Vidal Ceballos,[a,c] Tong Wang,[a] Shadman Khandaker,[a] Rinat R. Abzalimov,[a] Ankit Jain,[a,d] Shana Elbaum-Garfinkle [a,c] and Rein V. Ulijn [a-c]

[a] Advanced Science Research Center (ASRC) at the Graduate Center, City University of New York (CUNY), 85 St Nicholas Terrace, New York, New York 10031, United States.

[b] Department of Chemistry, Hunter College, City University of New York, 695 Park Avenue, New York, New York 10065, United States.

[c] Ph.D. Program in Chemistry, The Graduate Center of the City University of New York, New York, New York 10016, United States.

[d] Department of Chemistry and biochemistry, Brooklyn College, City University of New York, 2900 Bedford Avenue, Brooklyn, NY 11210, United States.

[e] Brooklyn College Cancer Center, Brooklyn College, The City University of New York

[f] Ph.D. Program in Chemistry, The Graduate Center of the City University of New York, New York, New York 10016, United States.

This PDF file includes:

Materials and Methods

Figs. S1, S2, S3, and S4

Other Supplementary Material for this manuscript includes:

Movie S1

Materials

Peptides were purchased from Genscript and were dissolved in 50 mM HCl solution to a concentration of 1 mg/mL to remove TFA. Solutions were then sonicated for 5 min and lypholized. The procedure was repeated 5 times. Final TFA content, <1% as confirmed by fluorine NMR. Tyrosinase from Mushroom and α-chymotrypsin was purchased from Sigma Aldrich.

Methods

Sample preperation

All peptides were dissolved to a stock solution of 5 mM and ATP was dissolved to 25 mM in 10 mM Tris HCl containing 15 mM KCl, 0.5 mM $MgCl_2$, and 0.2% (w/v) NaN_3 at pH 7 by vortex and sonication. Peptide solutions were added to ATP (equal charge ratio) and diluted with Tris buffer for an end concentration of 1 mM. For the oxidation reactions, tyrosinase was dissolved to 5 mg/mL in 100 mM phosphate buffer at pH 8, and diluted 10-fold when directly added to each peptide sample (final concentration: 0.5 mg/mL). Stock solutions of *f*-ATP were prepared in DI water, final concentration (0.5 mM).

Slide surface functionalization

Adapted from (Mountain et al. Biomacromolecules 2020, 21, 630–640). The cover slides were dipped in 0.5 M KOH solution in Isopropanol for 30 mins and washed with DI water. The slides were then left to dry in an oven overnight at 90 ºC. The slides were then cooled to room temperature and dipped into 3 mg/mL solution of N-(Triethoxysilylpropyl)-O-polyethylene oxide urethane in Toluene for 4 hours minimum. The slides were taken out and were wiped (with Kim wipes) with Isopropanol or acetone gently to removeany visible evaporation induced marks.

Optical microscopy

5 μl of each samples were pre-aged for 1 hour on a functionalized glass slide and then covered by a coverslip with a 1 mm spacing using double-sided adhesive tape between slides. Samples were then imaged using a Nikon Eclipse Ts2 micropscope equiped with a Lumenera Infinity lite B CMOS camera.

UV-Vis aborption measurements

Peptides were added to ATP and settled for 1 hour, the samples were centrifuged for 10 min at 15,000 rpm. The supernatant was collected and transferred onto another reaction tube. 25 μl of Tris buffer was then added to the droplets seperate from the biphase mixure. UV-vis spectra between 300 and 800 nm was collected for both phases before any addition of enzyme using a Hellma Tray Cell Cuvette for UV-Vis Analysis with a 1.0 mm path length. For oxidative measurements, equal amounts of tyrosinase (10-fold amount by volume) was added to each reaction tube and following 0, 1 and 3 hours of oxidation and polymerization, the absorption spectra were analyzed using Jasco V-660 spectrophotometer. The spectra was measured with a medium response and 2 nm intervals.

Matrix Assisted Laser Desorption/Ionization

Samples were prepped and left to oxidize for 24 h at room temperature. All samples were diluted to 100 μM using 10 mg/mL 3,5-Dimethoxy-4-hydroxycinnamic matrix and left to air dry on a MTP 384 target plate ground steel BC. MALDI was analyzed in linear mode.

Cryo-Transmission electron microscopy

Grids were purchased from Ted Pella, Inc. (Prod # 01895-F), which has lacey carbon support film. All grids were treated for 45 seconds in Fischione Nanoclean 1070 (70% power) with a mixture of Argon (75%) and Oxygen (25%). 5 μl of oxidized coacervate sample was aged directly on a lacey carbon grid covered with an additional thin layer of continuous carbon film overnight in a closed vial to avoid evaporation. The sample solution was plunged into liquid ethane that was pre-cooled by liquid nitrogen. The cryo-EM grids were then transferred to and stored in liquid nitrogen. The cryo-EM grid was transferred in liquid nitrogen into a Gatan 626 cryo-specimen holder that was then inserted into the microscope stage. The specimen temperature was maintained at about −170 °C during data collection. Cryo-EM imaging was performed in low dose mode of Titan Halo TEM operating at 300 kV and CETA (Thermo Fisher Scientific).

Fluorescence recovery after photobleaching

Oxidized sample was aged on a functionalized glass slide and covered by a coverslip with a 1 mm spacing. FRAP experiments were performed on a Marianas Spinning Disk confocal microscope (Intelligent Imaging Innovations) consisting of a spinning disk confocal head (CSU-X1, Yokagawa) on a Zeiss Axio Observer inverted microscope using a ×100/1.46 NA PlanApochromat oil immersion objective. A radius of 0.5 μm was bleached for 5 ms laser line from a solid state laser (LaserStack). Following recovery of the bleached area, excitation from the laser line was captured and collected with a 440/521/607/700-nm quad emission dichroic and 525/30-nm emission filter. Images were captured using a Photometrics Prime sCMOS camera with SlideBook 6 (Intelligent Imaging Innovations). ImageJ was used to export intensity traces. The final FRAP recovery curves are the average of three distinct droplet recovery curves. Excel was used to do photobleaching correction, normalizing, and averaging.

Confocal Microscopy

Oxidized sample was aged on a functionalized glass slide and covered by a coverslip with a 1 mm spacing. Imaging was performed using a Leica TCS SP8 STED 3X with 100x objective lens (with oil immersion). UV light laser (405 nm, 15% power) was used to observe intrinsic fluorescence following excitation and emission ranges (405 nm, 420-676 nm).

Liquid Chromotography-Mass Spectrometry

A 10 mg/mL stock solution of Chymotrypsin was prepared in 100 mM phosphate buffer at pH 8. For oxidized experiments, tyrosinase was diluted 10-fold when directly added to each peptide sample (1mM) and left to oxidize for 1 and 3 hours. Chymotrypsin (1 mg/mL) was then added to the solution containing oxidized product for 15 min The sample was then quenched 10-fold using 1:1 Water and Acetonitrile with 0.1% Formic Acid. LCMS analysis was performed using a Vanquish Flex LC (Thermo-Fisher) equipped with a Phenomenex Luna 100 Å 5 μm 50x2 mm C18(2) column with a flow rate of 0.2 mL/min, coupled to a Bruker MaXis II EDT mass spectrometer. Mass spectrometry data was collected in positive mode electrospray ionization (500 V) and collision energy of 2.0 eV. Samples were analyzed by flow injection analysis using an Acetonitrile-Water gradient with 0.1 % formic acid.

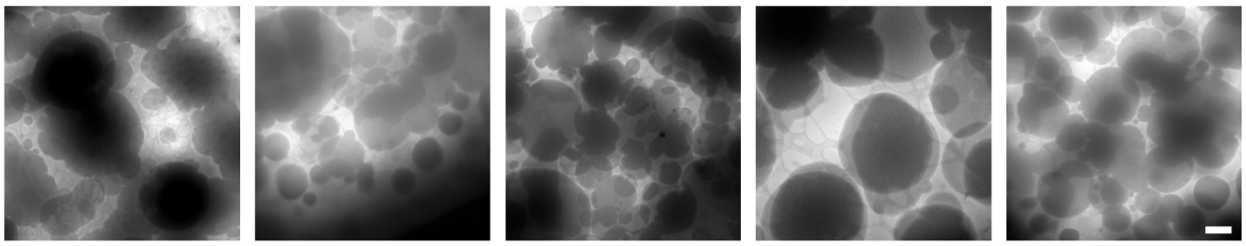

Figure S1 - Additional Cryo-TEM images of $DYFR_{9\ ox}$ coacervates. Scale bar = 2 μm

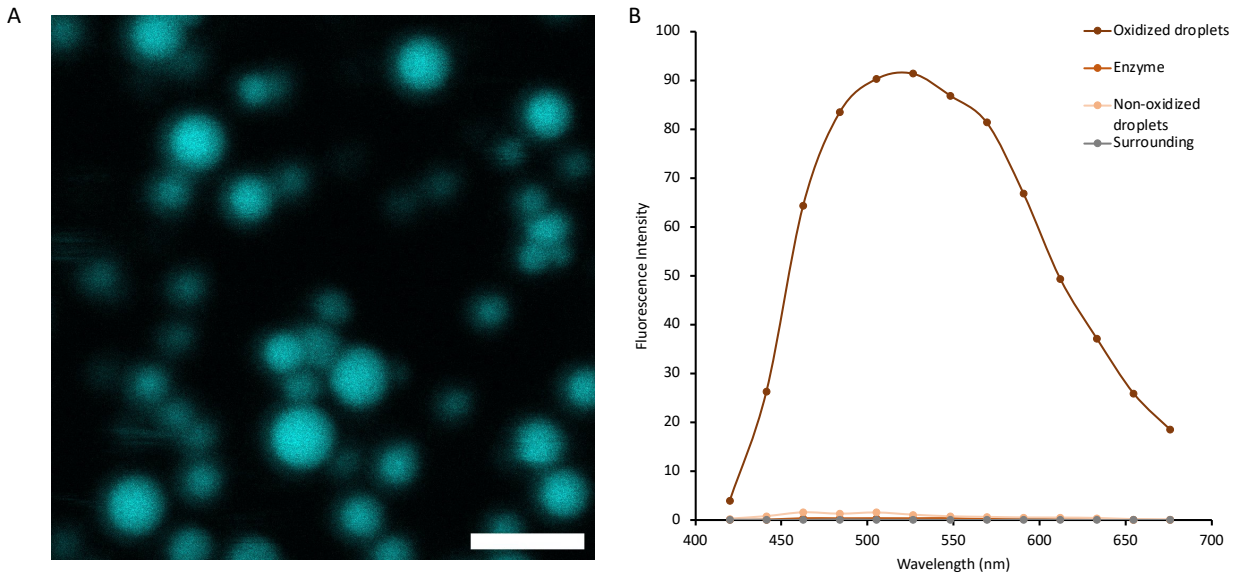

Figure S2 – Confocal Microscopy analysis of oxidized $DYFR_9$ + ATP intrinsic fluorescence. (A) Confocal microscopy image of $DYFR_{9\ ox}$ droplets. Scale bar = 2 μm (B) Normalized emission spectra at 420-676 nm measured from the oxidized droplets, surrounding background, non-oxidized droplets, and enzyme.

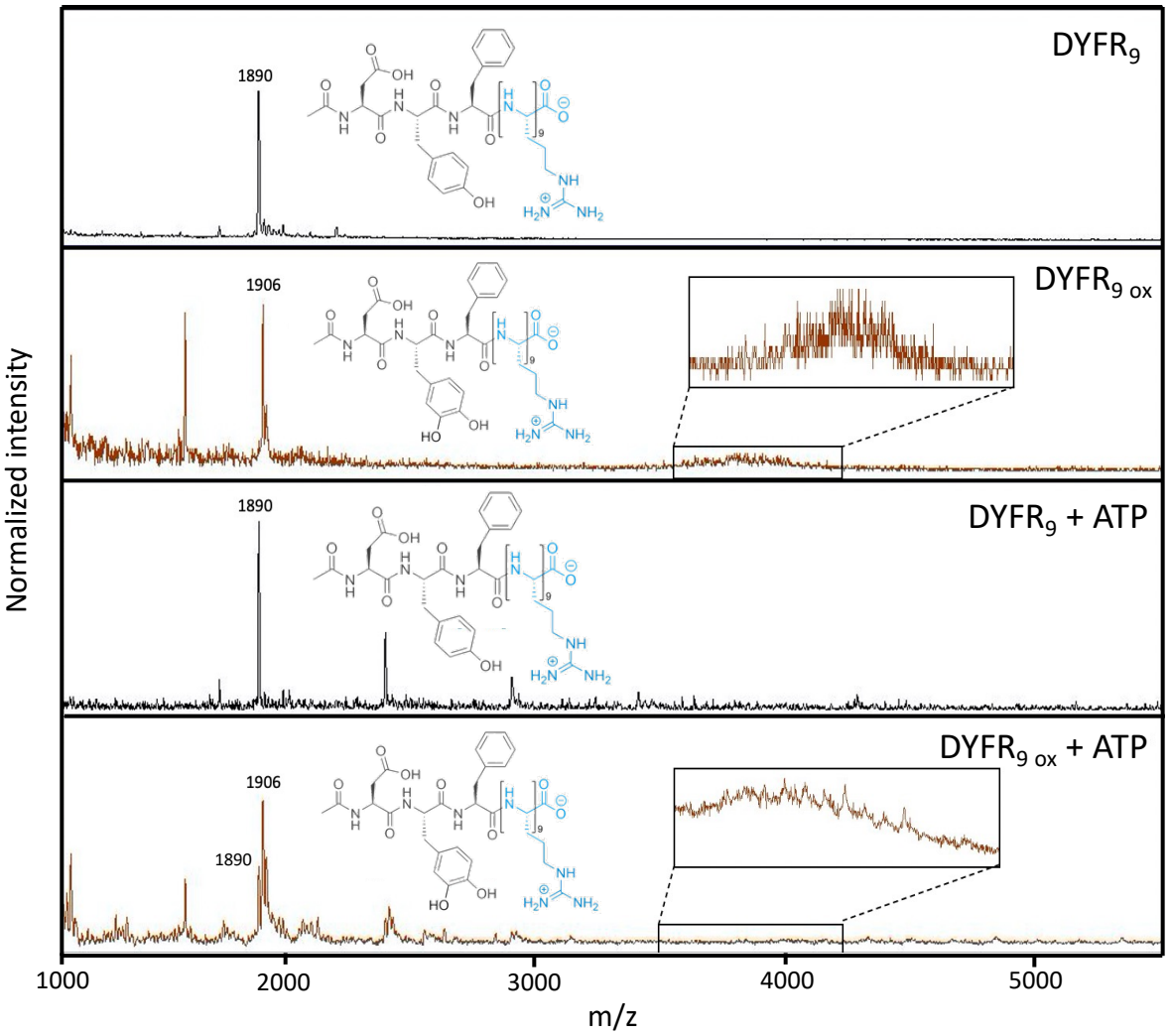

Figure S3 - Characterization of DOPA product in liquid droplets. MALDI spectra of $DYFR_9$, $DYFR_{9\ ox}$, $DYFR_9$ + ATP and $DYFR_{9\ ox}$ + ATP. Oxidized spectra are taken at t = 24h.

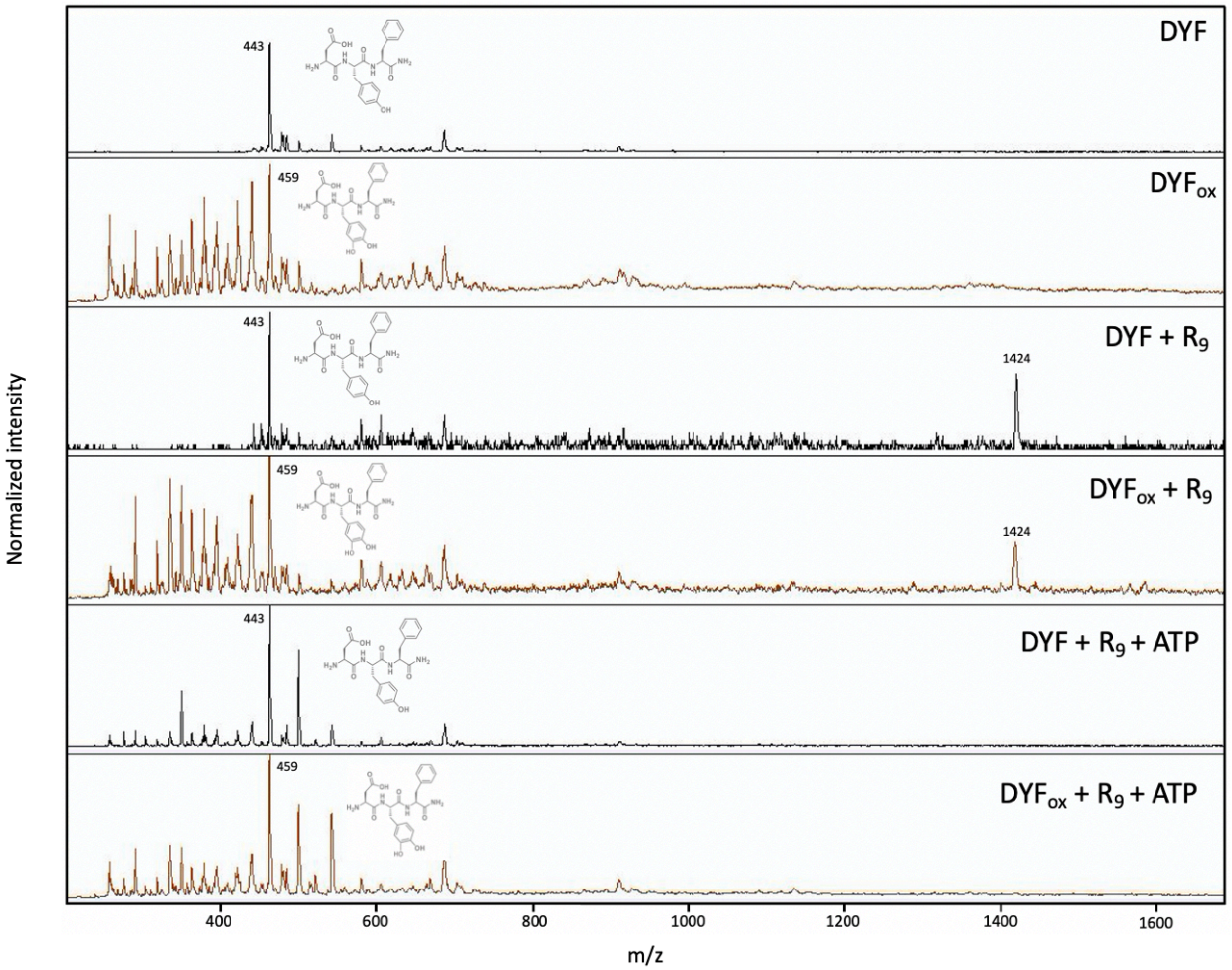

Figure S4 - Characterization of DOPA product in DYF controls. MALDI spectra of DYF, DYF $_{ox}$, DYF + R_9, DYF $_{ox}$ + R_9. In droplets: DYF + R_9 + ATP and DYF $_{ox}$ + R_9 + ATP. Oxidized spectra are taken at t = 24h.

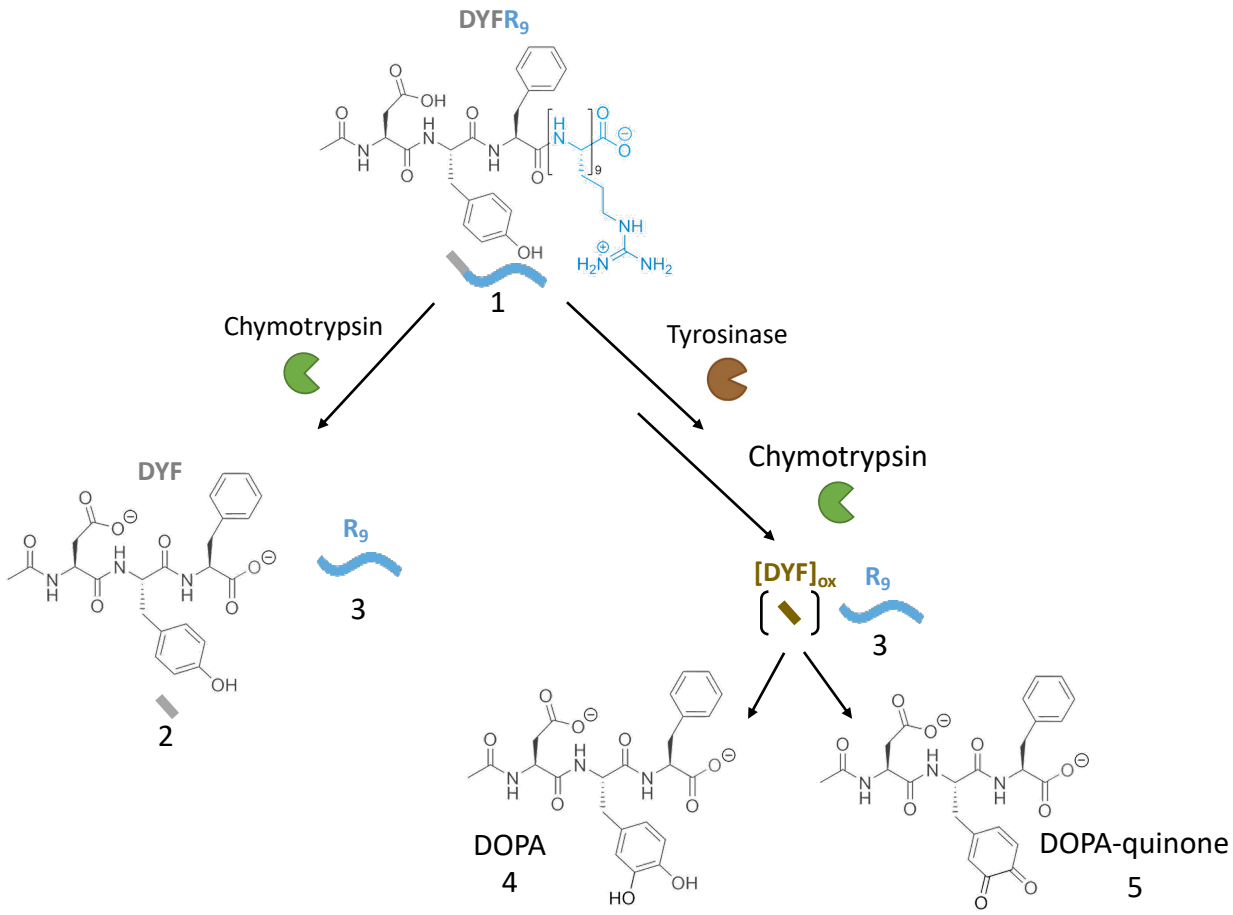

Figure S5 – Schematic showing the chymotrypsin digestion of Ac-DYFR$_9$ and Ac-DYFR$_{9\ ox}$.

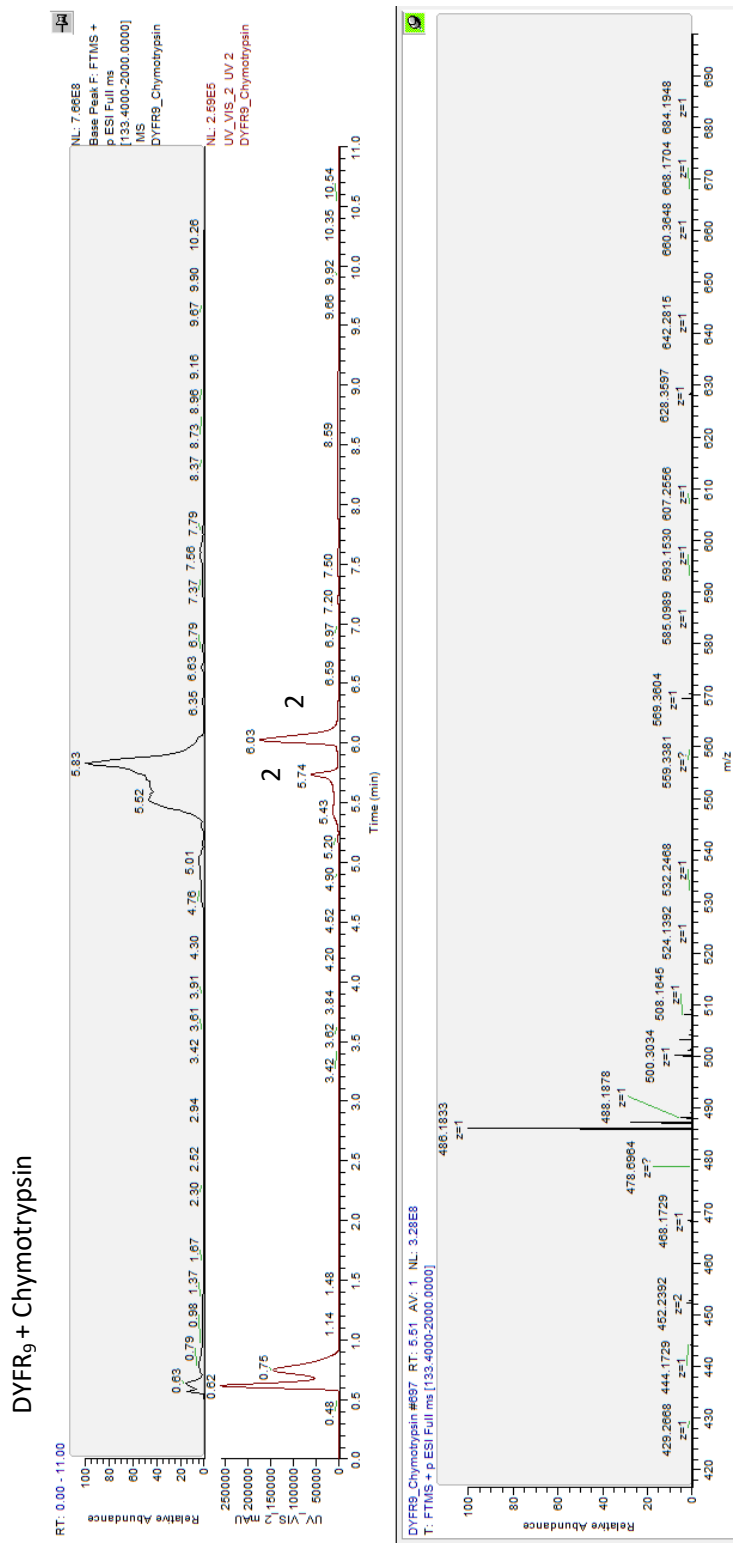

Figure S6 – EIC and analytical HPLC chromatogram of Ac-DYFR$_9$ after Chymotrypsin digestion and R$_9$ tail cleavage. We observed peak splitting due to sodium ion pairing effect. Mass spectra of RT 6.03 and 5.74 min expected 485.4760 m/z, found 486.1835 m/z [M/1+H], 508.1663 m/z [M/1+Na].

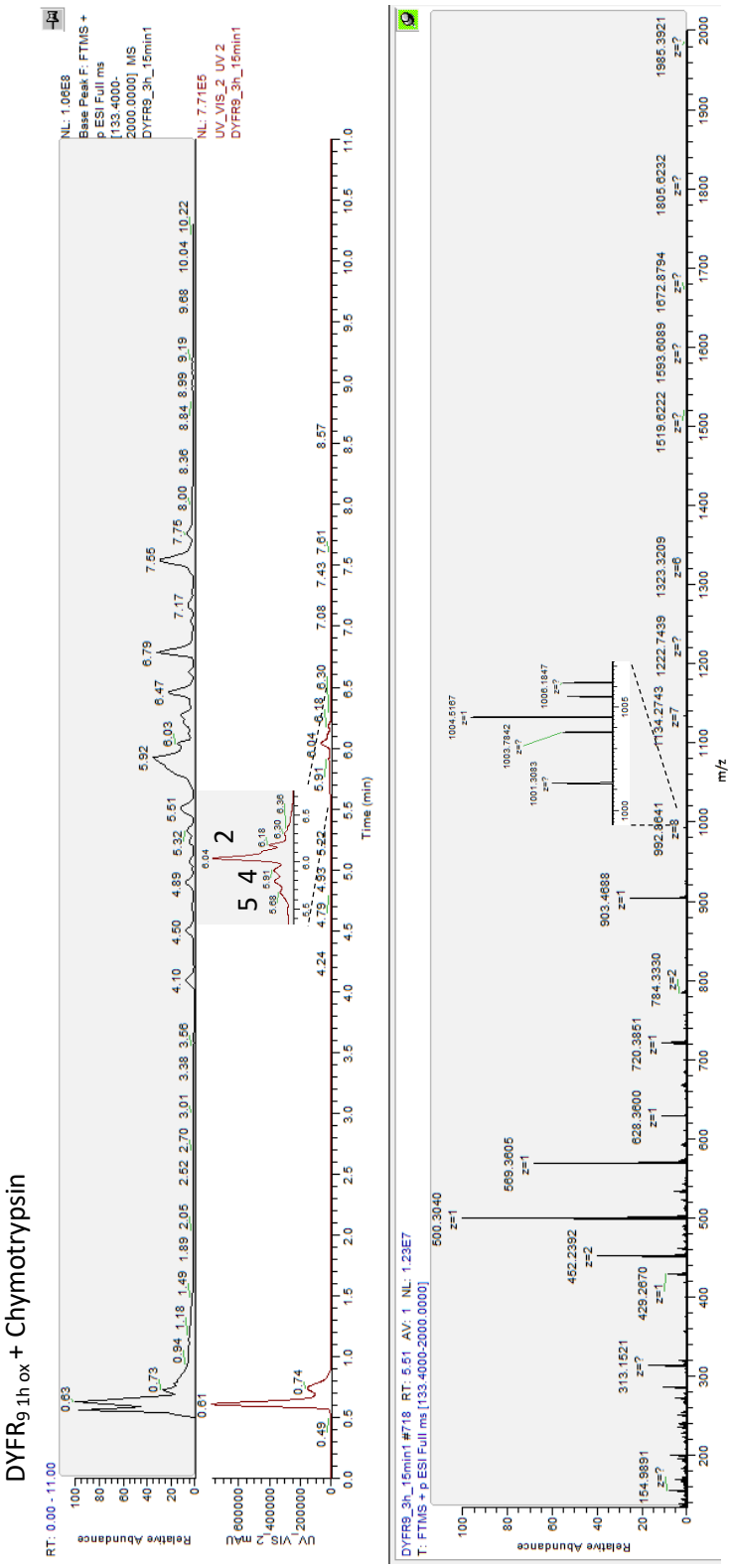

Figure S7 - EIC and analytical HPLC chromatogram of Ac-DYF 1 hour after oxidation, followed by Chymotrypsin digestion and R_9 tail cleavage. Mass spectra of RT [4] 5.91 min expected 502.1835 m/z, found 502.1805 m/z [M/1+H], 524.1619 [M/1+Na] and [5] 5.79 min expected 500.1835 m/z, found 500.1651 m/z [M/1+H], 522.1472 [M/1+Na].

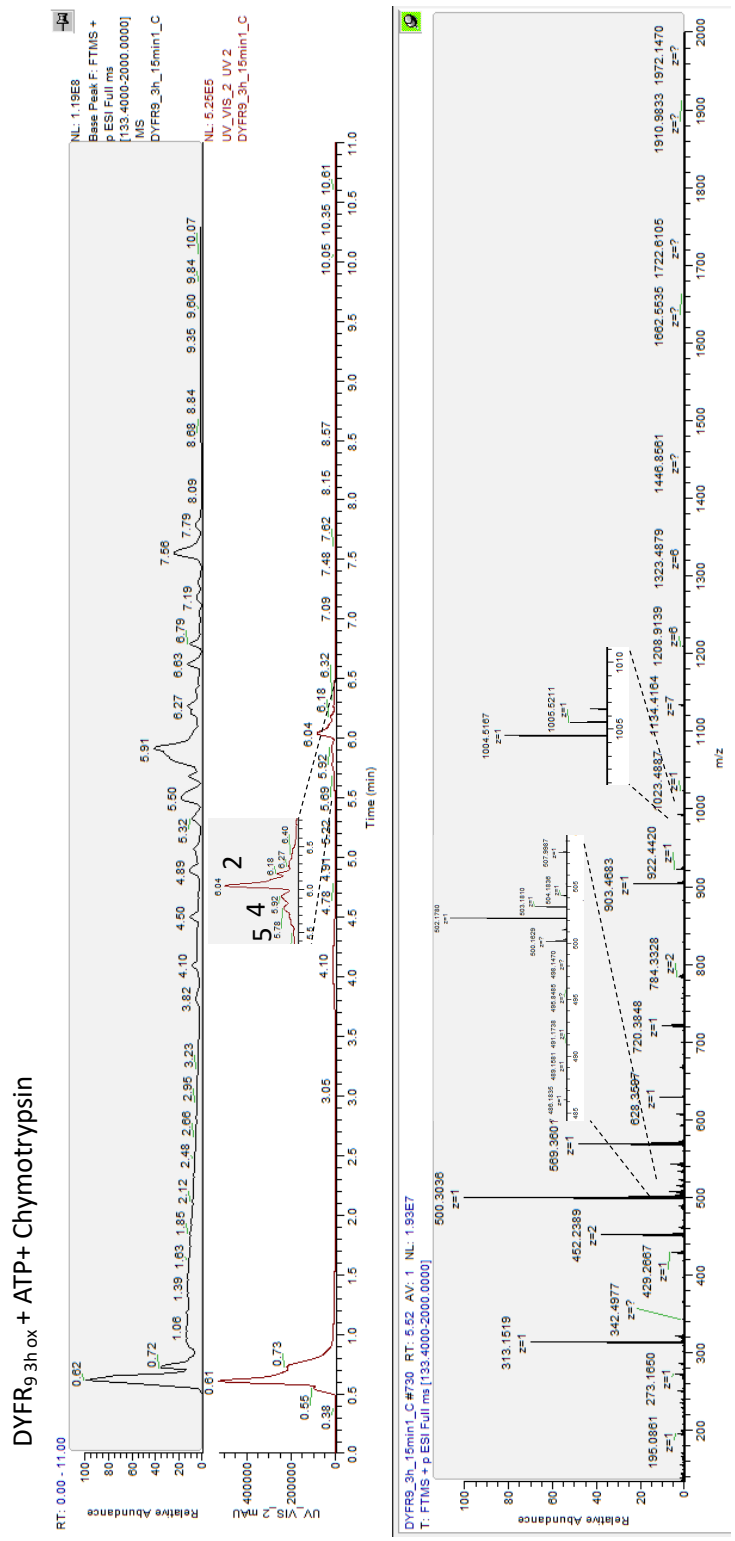

Figure S8 - EIC and analytical HPLC chromatogram of Ac-DYF 3 hours after oxidation in the presence of coacervates, followed by Chymotrypsin digestion and R_9 tail cleavage. Mass spectra of RT [4] 5.92 min expected 502.1835 m/z, found 502.1789 m/z [M/1+H], 524.1605 [M/1+Na] and [5] 5.79 min expected 500.1835 m/z, found 500.2690 m/z [M/1+H], 522.2534 [M/1+Na].