Supporting Information

Fish and Clips: A Convenient Strategy to Identify Tyrosinase Substrates with Rapid Activation Behavior for Materials Science Applications

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1. Materials

Cyan bromide, 2,2'-(ethylenedioxy)bis(ethylamine), α-Cyano-4-hydroxy-cinnamic acid (α-CHCA, 99%), formic acid (FA, ~98%) and succinic anhydride were purchased from Sigma Aldrich (Seelze, Germany). Acetonitrile (HPLC-MS grade), ethanol absolute (>99.7%) and hydrochloric acid (37%) were obtained from VWR chemicals (Philadelphia, USA). Sodium sulfate (≥99%) was recieved from Carl Roth GmbH (Karlsruhe, Germany). Sodium chloride (≥99%) was obtained from Acros Organics (Geel, Belgium). Hellmanex III was acquired from Hellma GmbH (Müllheim, Germany). Sodium hydroxide and Rhodamine B were obtained from Fluka analytical. All chemicals were used as received without further purification. All buffers and aqueous solutions were prepared with Milli-Q water.

1.1 Peptide Synthesis

N-α-Fmoc protected amino acids Fmoc-Ala-OH, Fmoc-Arg(Pbf)-OH, Fmoc-L-Asn(Trt)-OH, Fmoc-Cys(Trt)-OH, Fmoc-Gly-OH, Fmoc-His(Trt)-OH, Fmoc-Lys(Boc)-OH, Fmoc-L-Met-OH, Fmoc-Pro-OH, Fmoc-Ser(*t*Bu)-OH, Fmoc-Tyr(Boc)-OH, Fmoc-L-Phe-OH as well as coupling reagents 2-(1H-benzotriazole-1-yl)-1,1,3,3-tetramethyluronoium hexafluorophosphate (HBTU), benzotriazole-1-yl-oxy-tris-pyrrolidino-phosphonium hexafluorophosphate (PyBOP), resin Fmoc-Rink Amide AM resin (loading 0.59 mmol/g) and solvents *N*-methyl-2-pyrrolidone (NMP, 99.9%, peptide synthesis grade) as well as dichloromethane (DCM, peptide symthesis grade) were ordered from IRIS Biotech GmbH (Marktredwitz, Germany). NMP was filtrated and DCM was distilled from CaH_2 prior to use. Aminomethyl ChemMatrix resin (loading 0.1 mmol/g) was purchased from Sigma Aldrich (Seelze, Germany). TentaGel S RAM (loading 0.23 mmol/g) and TentaGel PAP resin (loading 0.24 mmol/g, Mw=3200) were obtained from Rapp Polymere GmbH (Tübingen, Germany). *N,N*-diisopropyl ethylamine (DIPEA, peptide synthesis grade), piperidine (peptide synthesis grade), 2,5-dihydroxybenzoic acid (99%) were purchased from Acros Organics (Geel, Belgium). DIPEA was distilled prior to use. Triethylsilane (TES, 98+ %) was obtained from Alfa Aesar (Karlsruhe, Germany). Trifluoroacetic acid (TFA, peptide grade) from Acros Organics (Geel, Belgium) was distilled prior to use. Acetic anhydride was purchased from Carl Roth (Karlsruhe, Germany).

1.2 Enzymatic assay and reactions

Tyrosinase (>1000 units/mg) from mushroom was purchased from Sigma Aldrich (Seelze, Germany). Enzyme lyophilisates as well as solutions were stored at -20 °C. Lyophilisates were dissolved in potassium phosphate buffer (50 mM, pH 6.5) prior to use. L-Tyrosine (≥98%), potassium phosphate monobasic (≥98%) and potassium phosphate dibasic (≥98%) were obtained from Sigma Aldrich (Seelze, Germany). *L*(+)-ascorbic acid (≥99%, Roth, Karlsruhe, Germany) and *L*(+)-ascorbic acid sodium salt (≥99%, Fluka) were used without further purification.

2. Instrumentation

^{1}H nuclear magnetic resonance spectra (^{1}H-NMR) were recorded on a Bruker AV 500 spectrometer at 500 MHz in TFA-d1 at room temperature.

UV/vis spectroscopy was carried out on a EonC Microplate Spectrophotometer with cuvette port (BioTek, Bad Friedrichshall, Germany) using quartz cuvettes for enzymatic assay and Greiner UV-Star 96 well microplates for kinetic measurements.

Preparative HPLC was conducted on a Shimadzu prominence system equipped with a CBM-20A communications bus module, a LC-20AP preparative liquid chromatograph pump unit, a SPD-10A UV-VIS detector and a FRC-10A fraction collector. Chromatographic separation was conducted on a Synergi Fusion-RP column (250 x 21.2 mm, phenomenex, Germany). As solvent, mixtures of solvent A/solvent B (solvent A: 99.9% Milli-Q H_2O - 0.1% FA; solvent B: 99.9% acetonitrile - 0.1% FA) were used with a flow rate of 22.0 mL/min.

MALDI-TOF mass spectrometry was performed on an autoflex III smartbeam system (Bruker, USA) with matrix assisted laser desorption/ionization and time of flight detector. On the sample plate, 2 µL of sample were mixed with 1 µL matrix solution consisting of 7 mg/mL α-cyano-4-hydroxy-cinnamic acid in Milli-Q-water/acetonitrile (1:1, v/v) with 0.1 % TFA. Samples were air-dried at ambient temperature. Measurements were performed in linear positive mode.

MALDI-TOF-MS/MS measurements were conducted on a 5800 MALDI-TOF/TOF system (AB Sciex , USA). Samples were mixed in a 1:1 ratio with matrix solution, consisting of 7 mg/mL α-cyano-4-hydroxy-cinnamic acid in Milli-Q-water-acetonitrile (1:1, v/v) with 0.1 % TFA, on the sample plate and air dried at ambient temperature. Measurements were performed in reflector positive mode, with 4000 shots and laser intensity of 3200 for MS and 12500 shots and laser intensity of 4300 for MS/MS.

UPLC-QMS was carried out on an Acquity UPLC H-class system (Waters, USA) with a PDA and QDa detector. Acquity UPLC BEH C18 columns (2.1 x 100 mm, 2.1 x 50 mm, Waters, USA) were used for chromatographic separation with a solvent mixture of solvent A/solvent B (solvent A: 99.9% Milli-Q H_2O - 0.1% FA; solvent B: 99.9% acetonitrile - 0.1% FA) and a flow rate of 0.5 mL/min.

Quartz crystal microbalance measurements were conducted on a Q-sense Explorer E1 single-sensor QCM-D module with dissipation combined with a QE 401 Electronic Unit (Biolin Scientific AB, Sweden) and equipped with a multichannel pump (IPC Ismatec SA, Switzerland). Piezoelectric sensor crystals coated with 100 nm aluminum oxide (QSX 309, Q-Sense, Sweden) were used for adsorption measurements.

Fluorescence microscopic investigations and images were made using a Carl Zeiss Axio Observer.A1 microscope running on AxioVs40 (Ver. 4.8.2.0) Software equipped with a HXP 120 C fluorescence illumination system. Fluorescence was observed using a Cy3 filter (570 – 650 nm). All fluorescence images were recorded with an exposure time of 60 ms at 20% lamp brightness.

3. Experimental details

3.1 Automated peptide synthesis

Peptides were synthesized following standard ABI-*Fastmoc* protocol (single coupling with capping) with NMP as solvent using standard Fmoc-amino acid derivatives. As solid support, Fmoc-Rink Amide resin (loading 0.59 mmol/g, 0.1 mmol) was used for peptides and TentaGel PAP resin (loading 0.24 mmol/g, 0.1 mmol) was used for the synthesis of peptide-PEG conjugates. Synthesis was performed on an automated *ABI 433a* peptide synthesizer (Applied Biosystems, Foster City, USA). Fmoc-amino acid coupling was facilitated by HBTU/DIPEA. For the peptides, resin was transferred to a syringe reactor and subsequently washed with dichloromethane prior to final Fmoc removal. Peptide-PEG conjugates were transferred after final Fmoc removal. Products were cleaved from the solid support with a mixture of 95:4:1 vol-% TFA/H_2O/TES for 3 h and resulted in fully deprotected peptide-polymer conjugates and deprotected Fmoc-carrying peptides. The resin was filtered, washed with TFA and the collected supernatants were concentrated in vacuo. The product was isolated by precipitation with diethyl ether and subsequent centrifugation, which was repeated at least three times after redissolving in TFA. Purified products were obtained by lyophilization from Milli-Q water. Peptides were further purified using preparative HPLC and collected fractions were lyophilized. Afterwards Fmoc removal was performed in a solution of 20% piperidine in dichloromethane, followed by evaporation of the solvent using N_2, precipitation in diethyl ether with subsequent centrifugation and discarding of the supernatant. Purified peptides were obtained by lyophilization from Milli-Q water.

In total, peptides Pep_1 (SSYGS), Pep_2 (HSYHG), Pep_3 (NGYHN), Pep_4 (NGYSS), Pep_5 (SYYKS), Pep_6 (SYYHP), Pep_7 (SPYAS), Pep_8 (NSYHS), Pep_9 (KYYHP), Pep_{10} (PRYPR) Pep_{11} (AAYAP) and peptide-PEG conjugates $(Pep_2)_3$-PEG and $(Pep_4)_3$-PEG were obtained by this method.

3.2 Manual peptide synthesis

Manual synthesis followed standard Fmoc protocol solid phase peptide synthesis with NMP as solvent using Fmoc-amino acid derivatives. As solid support, Aminomethyl-ChemMatrix resin (loading 1.0 mmol/g) was used. Coupling reactions were performed for 1 h using 5 eq of amino acid, 5 eq PyBOP and 10 eq of DIPEA. The first coupling step to the resin was conducted for 4 h using only 3 eq of the respective reagents. After each coupling step unreacted amines were acetylated using a solution of 4.99 vol% acetic anhydride, 2.25 vol% DIPEA, 0.19 wt% HOBt in NMP, followed by Fmoc removal with 20 vol% piperidine in NMP for 2 x 5 min. The filtrate

was used for UV monitoring to quantify the reaction rate of each coupling step showing quantitative yields. The resin was washed with 5 x NMP, 5 x DCM, 5 x NMP in between every step. Protection groups were removed by suspending the resin in a mixture of 95:4:1 (v/v) TFA/H_2O/TES for 3 h followed by final washing with DCM and drying of the beads *in vacuo*. Peptides $Testpep_1$ (Ac-YGG-GGGM) and $Testpep_2$ (FGG-GGGM) were obtained by this method.

3.3 Split and mix peptide library

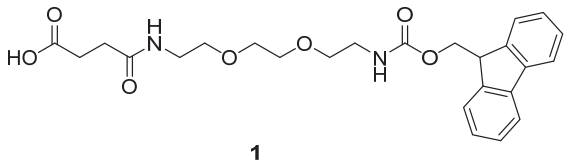
3.3.1 Synthesis

The synthesis of the library was done according to the manual peptide synthesis protocol using Aminomethyl-ChemMatrix resin (loading 1.0 mmol/g) in a 250 µmol scale using the split and mix procedure. The library contained approximately 230 000 beads with an average of 39 beads per sequence. At first a cleavable linker sequence (GGGM) was synthesized on the whole resin. Then followed two combinatorial steps where the resin was divided into 9 aliquots by suspending the resin beads evenly in DCM and seperating equal volumes into 9 different syringe reactors. Every aliquot was then coupled to a different amino acid (A,G,H,K,N,P,R,S,Y) and all aliquots were combined for the acetylation and Fmoc removal steps before they were divided again for the second coupling step. During synthesis coupling steps were carried out for at least 3.5 h to guarantee quantitative yields. After two combinatorial coupling steps the third one of the combinatorial 5-mer was performed with the whole amount of resin with amino acid Y. Afterwards again two coupling steps were carried out with 9 and 8 amino acids, respectively because the last coupling was done without Y to ensure this amino acid is missing on the n-terminal position.

3.3.2 Screening and identification of selected beads

After activation (cf. 3.7) the resin was transferred to a petri dish, suspended in water and placed under a fluorescence microscope. Fluorescent beads were each selected manually and placed into a 0.2 mL PCR tube. Cleavage from the resin was then carried out by adding 20 µL of CNBr solution (20 mg/mL in 0.1 M HCl) and leaving the mixture to react overnight. Afterwards the solution was frozen and lyophilized. The residue was solubilized in ACN/Milli-Q water (1:1, v/v) + 0.1% TFA for analysis with MALDI-TOF-MS/MS.

3.4 Fmoc-4-(2-(2-(2-aminoethoxy)ethoxy)ethylamino)-4-oxobutanoic acid (1)



1

10.0 mL (68.5 mmol, 1 eq) 2,2'-(ethylenedioxy)bis(ethylamine) were added to a round-bottom flask and dissolved in 340 mL acetonitrile followed by the dropwise addition of 6.83 g (68.5 mmol, 1 eq) succinic anhydride dissolved in 170 mL acetonitrile to the solution. The reaction was stirred for 2.5 h and a waxy substance was formed. The supernatant organic solvent was decanted and the residue was dissolved in 700 mL ACN/Milli-Q water (1:1, v/v) and cooled to 0 °C. The pH was adjusted to 8 with 10% aquaeous $NaHCO_3$ solution followed by the dropwise addition of 22.98 g (88.8 mmol, 1.3 eq) Fmoc-Cl in 70 mL acetonitrile. After a second pH adjustment, the solution was stirred overnight at RT and subsequently concentrated *in vacuo*. The remaining product was adjusted to pH 2 with 1 M HCl, then extracted with EtOAc 4 times and the combined organic layer was washed twice with water. The organic layer was dried over Na_2SO_4, filtered and concentrated *in vacuo*. The residue was purified using column chromatography (EtOAc -> EtOAc : MeOH : TFA, 9:1:0.1, v/v). 13.94 g (43%) of the pure product were obtained as a clear oil.

^{1}H-NMR (500 MHz, $CDCl_3$): δ = 7.76 (d, *J* = 7.53 Hz, 2H), 7.55 (d, *J* = 7.57 Hz, 2H), 7.40 (t, J^1 = 7.46 Hz, J^2 = 7.46 Hz, 2H), 7.31 (t, J^1 = 7.33 Hz, J^2 = 7.33 Hz, 2H), 4.53 - 4.37 (m, 2H), 4.28 - 4.18 (m, 1H), 3.65 - 3.25 (m, 12H), 2.76 - 2.63 (m, 2H), 2.60 - 2.48 (m, 2H).
^{13}C -NMR (125 MHz, $CDCl_3$): δ = 177.6 (s, CO), 159.3 (s, CO), 158.4 (s, CO), 143.7 (s, C_{arom}), 141.4 (s, C_{arom}), 127.9 (s, CH_{arom}), 127.2 (s, CH_{arom}), 124.9 (s, CH_{arom}), 120.1 (s, CH_{arom}), 70.2 (s, CH2), 70.1 (s, CH2), 69.9 (s, CH2), 69.1 (s, CH2), 67.9 (s, CH2), 47.2 (s, CH), 41.3 (s, CH2), 39.9 (s, CH2), 30.8 (s, CH2), 29.9 (s, CH2).

ESI-MS: m/z calc. (monoisotopic) $[M+H]^+$ = 471.21, $[M+Na]^+$ = 493.19; found $[M+H]^+$ = 471.17, $[M+Na]^+$ = 493.17.

3.5 Preparation of the fluorescent probe

The synthesis of the fluorescent probe was performed according to the manual peptide synthesis protocol using polystyrene Fmoc-Rink amide resin (loading 0.59 mmol/g). Coupling steps of amino acids were performed as described. Coupling steps of the spacer molecule **1** and Rhodamine B were each carried out twice for 1 h before removal of Fmoc protection group was performed. The product was cleaved from the solid support with a mixture of 95:4:1 (v/v) TFA/H_2O/TES for 3 h and resulted in fully deprotected products. The product was isolated by precipitation with diethyl ether and subsequent centrifugation, which was repeated at least three times after redissolving in MeOH. Purified products were obtained by lyophilization from gegassed Milli-Q water.

3.6 Tyrosinase activity assay

Prior to use of enzyme, an activity assay was performed using UV spectroscopy based on the method of *Duckworth* and *Coleman*[1]. The absorbance from the oxidation of tyrosine to dopaquinone is monitored at 280 nm over a period of 20 min at 25 °C using a 3 mL quartz cuvette. The assay solution contained 1 mL of potassium phosphate buffer (50 mM, pH 6.5), 1 mL of tyrosine solution (1 mM in Milli-Q water), 0.9 mL of Milli-Q water and tyrosinase stock solution (in 50 mM potassium phosphate buffer, pH 6.5) and filled up to 3 mL with potassium phosphate buffer. Enzyme stock solution was added immediately before starting the measurement. The activity was calculated using the average slope of the 3 minute interval with a maximum slope in absorbance Δ_{A280} according to equation 1.

$$enzyme\ activity\ [Units] = \frac{\Delta_{A280}}{0.001} \qquad \text{(eq. 1)}$$

3.7 Enzymatic activation of resin-bound peptides

Approximately 1 mg of resin (0.44 µmol) was swollen in Milli-Q water in a syringe reactor for at least 1 h. Afterwards, the resin was suspended in 1 mL of a solution of 100 Units tyrosinase, 13.7 µg sodium ascorbate (69.3 nmol) as an activation cofactor and 0.5 eq (0.21 mg, 0.22 µmol) of the fluorescent probe for 5 min. After the incubation period the resin was filtered and washed with 3 x MeOH and 2 x Milli-Q water.

3.8 Enzymatic activation reactions for kinetic studies in solution

For the activation of peptides to the corresponding quinone, the peptide was dissolved in potassium phosphate buffer (17 mM, pH 6.5) to a final concentration of 0.25 µmol/mL. Final reaction volume was reached after addition of an activation solution consisting of 33 µL ascorbic acid (2.1 mM in potassium phosphate buffer, 50 mM, pH 6.5) and 100 U tyrosinase (in potassium phosphate buffer, 50 mM, pH 6.5, volume varying depending on the activity assay) per mL of final reaction solution. The activation solution was added immediately at the beginning of the UV kinetic measurement.

Activation of peptides to the corresponding catechol was carried out under the same conditions, only ascorbic acid solution was replaced with a solution of sodium ascorbate (210 mM in potassium phosphate buffer, 50 mM, pH 6.5).

3.9 Quartz crystal microbalance

The piezoelectric sensor crystals coated with 100 nm aluminum oxide (QSX 309, Q-Sense, Sweden) were cleaned with 2% Hellmanex III solution (in Milli-Q water) for 15 - 30 min and ethanol in an ultrasonic bath for 10 min prior to use. Subsequently, the sensors were thoroughly washed by Milli-Q water and dried under compressed air flow. Finally, crystals were cleaned by air plasma in a ZEPTO plasma cleaner (diener electronic GmbH, Germany) for 3 min at 75 W. The sensors were mounted into the QCM flow chamber and incubated with degassed buffer using a flow rate of 100 µL/min until the frequency signals were constant (1-3 h). The measurement was started and sample solutions were pumped into the flow chamber. Experiments were performed at 22° C in a stop-flow mode, and overtones 3, 5, 7, 9, 11 and 13 were recorded. The third overtones of all experiments were used for evaluation of the frequency shift.

4. List of identified peptides

4.1 Sequences from highly fluorescent beads

	Sequence	Ion Score	Mass (expt)	Occurrence
1	KGYGYGGGM	86	840.3327	1
2	SPYASGGGM	82	777.3127	1
3	GPYKYGGGM	82	881.3672	1
4	PSYASGGGM	81	777.2827	1
5	SPYSAGGGM	81	777.2827	1
6	SSYAAGGGM	80	751.2627	1
7	NGYSSGGGM	80	780.2427	1
8	KSYPNGGGM	79	862.3612	1
9	SYYGPGGGM	77	839.2927	1
10	SYYHYGGGM	77	986.3633	1
11	NGYYGGGGM	77	827.2833	1
12	NGYPYGGGM	76	866.3027	1
13	SGYGNGGGM	76	750.2327	1
14	ASYNSGGGM	75	795.28	1
15	NNYPHGGGM	74	897.3127	1
16	SPYNYGGGM	74	897.3292	1
17	SAYPSGGGM	73	777.2827	1
18	GAYGSGGGM	73	707.2227	1
19	NSYHPGGGM	73	870.3527	1
20	NNYNGGGGM	73	834.2727	1
21	SNYHYGGGM	73	937.3254	1
22	SYYHPGGGM	73	920.3585	1
23	GHYSHGGGM	73	854.3096	1
24	NPYASGGGM	72	804.2727	2
25	SNYNAGGGM	72	821.3027	1
26	NPYSGGGGM	72	791.3228	2
27	NAYGNGGGM	72	792.26	1
28	SYYNYGGGM	71	962.2927	1
29	NPYSYGGGM	71	896.3927	1
30	SNYYSGGGM	70	886.2727	1
31	SAYANGGGM	70	778.2627	1
32	SYYKSGGGM	70	900.3527	1
33	NGYAHGGGM	70	838.3285	1
34	SHYPYGGGM	68	919.3427	1
35	GNYNNGGGM	68	835.2853	1
36	KNYKHGGGM	68	943.4464	1
37	NHYAGGGGM	67	814.3327	1
38	SSYYGGGGM	67	829.2827	1
39	SHYSSGGGM	67	834.2944	1
40	SAYNGGGGM	66	765.2566	1
41	HGYANGGGM	66	815.2944	1

42	APYAYGGGM	65	837.3627	1
43	SGYYGGGGM	65	800.2626	1
44	AGYGYGGGM	65	784.29	1
45	NSYSYGGGM	65	887.3127	1
46	NSYAGGGGM	64	765.25	1
47	NPYASGGGM	64	805.3042	2
48	SYYSNGGGM	64	887.3057	1
49	ANYHPGGGM	64	855.3635	1
50	GGYYSGGGM	64	800.2806	1
51	SNYYPGGGM	63	896.3027	1
52	NNYSSGGGM	63	837.2927	1
53	GNYNPGGGM	63	817.3227	1
54	HSYHNGGGM	63	910.3327	1
55	SPYNGGGGM	63	791.3185	1
56	HSYHGGGGM	62	854.3194	2
57	HSYHGGGGM	62	854.3099	2
58	ASYPAGGGM	61	761.2727	1
59	SPYAPGGGM	61	787.3427	1
60	SAYGHGGGM	61	788.2812	1
61	NYYNHGGGM	60	964.3562	1
62	ANYSSGGGM	60	795.27	1
63	PHYNYGGGM	60	947.3735	1
64	HHYGSGGGM	59	854.3254	1
65	NSYYHGGGM	59	937.3393	1
66	NAYSNGGGM	59	822.29	1
67	NGYYYGGGM	59	933.3404	1
68	GGYHAGGGM	58	757.2527	1
69	NAYPGGGGM	58	775.27	1
70	SNYGSGGGM	58	781.27	1
71	PSYHYGGGM	58	920.3559	1
72	NYYYGGGGM	58	933.3417	1
73	NGYHNGGGM	58	858.3173	1
74	HPYGPGGGM	57	823.3027	1
75	NYYSNGGGM	57	914.3123	1
76	NGYSYGGGM	57	857.2897	1
77	ASYNYGGGM	56	871.2991	1
78	NGYGAGGGM	56	735.26	1
79	SHYGPGGGM	56	814.3102	1
80	SAYRPGGGM	55	846.3527	1
81	GHYHYGGGM	55	930.3743	1
82	HHYYHGGGM	55	1010.3743	1
83	NPYGPGGGM	54	824.3383	1
84	NYYGPGGGM	53	866.3527	1
85	GAYNYGGGM	51	841.2916	1
86	NNYAGGGGM	51	792.28	1

87	SAYHGGGGM	50	787.3227	1
88	RHYHSGGGM	50	952.4227	1
89	SYYPPGGGM	50	880.3229	1
90	NHYNGGGGM	48	858.3429	1
91	NPYSGGGGM	48	791.33	2
92	SNYNRGGGM	45	906.4027	1
93	NGYNSGGGM	44	808.2806	1
94	NHYHRGGGM	42	980.4208	2
95	NHYHRGGGM	41	979.4627	2
96	NYYHSGGGM	41	937.3875	1
97	NNYSPGGGM	38	847.2809	1
98	SSYHAGGGM	36	866.3106	1
99	SSYGPGGGM	36	764.27	1
100	NNYASGGGM	35	822.33	1
101	SSYGSGGGM	34	754.25	2
102	SSYGSGGGM	32	753.3027	2
103	NAYHSGGGM	30	845.3556	1
104	HHYSGGGGM	29	854.3499	1

4.2 Sequences from low fluorescent beads

	Sequence	Ion Score	Mass (expt)	Occurrence
1	KPYPGGGGM	80	815.3792	1
2	KAYSAGGGM	78	793.37	1
3	PAYNGGGGM	73	775.31	1
4	KPYGHGGGM	69	855.3776	1
5	KAYPAGGGM	67	803.3782	1
6	AAYHPGGGM	66	812.3409	1
7	KYYHPGGGM	65	961.4282	2
8	KYYRAGGGM	65	954.4636	1
9	KYYHPGGGM	65	961.4282	2
10	GYYKAGGGM	59	855.4167	1
11	AAYAPGGGM	54	746.3	1
12	ASYYRGGGM	53	913.3975	1
13	PRYPHGGGM	50	923.46	1
14	PSYKAGGGM	49	819.3774	1
15	AAYKNGGGM	49	820.3688	1
16	KKYRAGGGM	44	919.4302	1
17	PRYPRGGGM	43	942.4791	1
18	RKYHPGGGM	34	954.5063	1
19	RPYRHGGGM	34	982.4987	1
20	KSYNNGGGM	26	879.3665	1

5. Compound Characterization

5.1 Pep$_1$ (SSYGS)

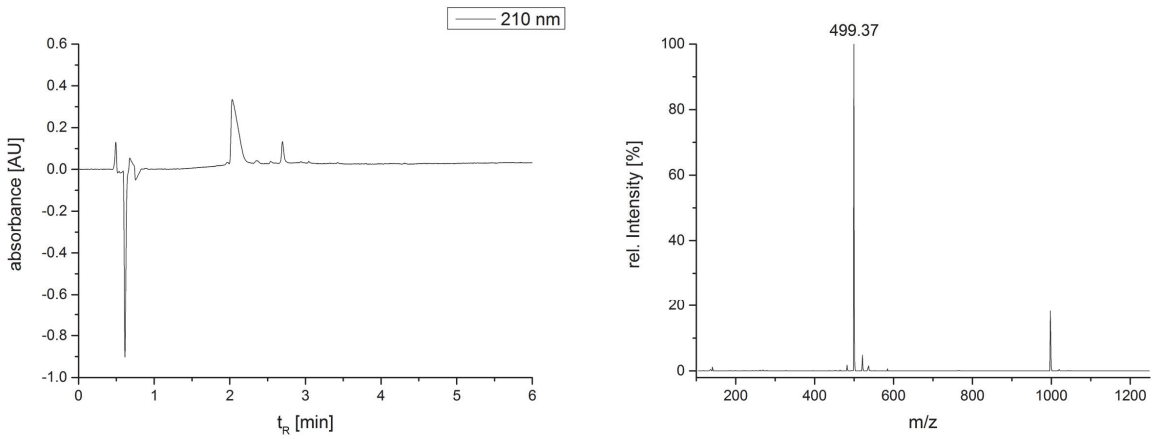


Figure S1. UPLC-MS analysis of Pep$_1$ (SSYGS), left: UV chromatogram at 210 nm, right: ESI-MS spectrum.

UV/VIS: t_R = 2.03 min. 88 % purity.

ESI-QMS: m/z calculated (average): $[M+H]^+$ = 499.50; found: $[M+H]^+$ = 499.37.

5.2 Pep$_2$ (HSYHG)

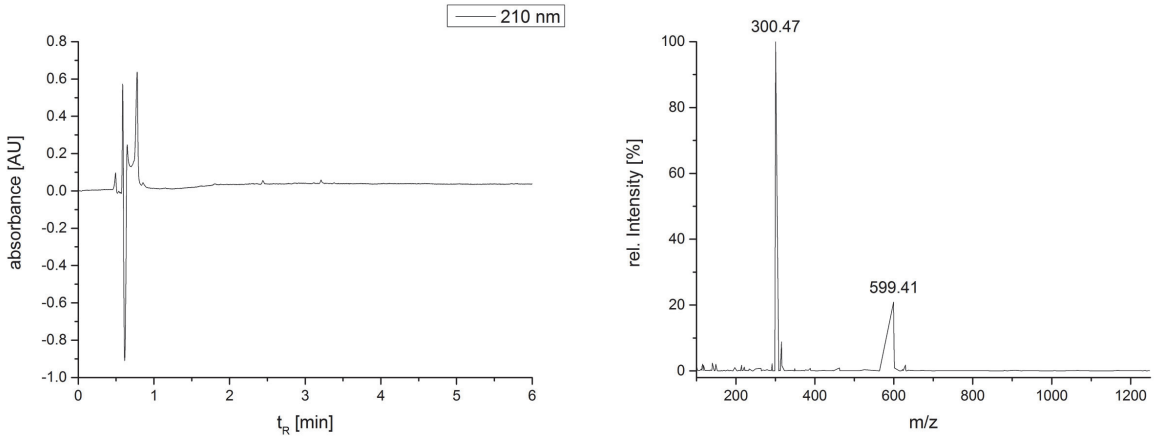


Figure S2. UPLC-MS analysis of Pep$_2$ (HSYHG), left: UV chromatogram at 210 nm, right: ESI-MS spectrum, t_R = 0.59 min.

UV/VIS: t_R = 0.59 min and 0.78 min. 87 % purity.

ESI-QMS: t_R = 0.59 min; m/z calculated (average): $[M+H]^{1+}$ = 599.63. $[M+2H]^{2+}$ = 300.32; found: $[M+H]^+$ = 599.41. $[M+2H]^{2+}$ = 300.47.

t_R = 0.78 min; m/z calculated (average): $[2M+Na+H]^{2+}$ = 610.62. found: $[2M+Na+H]^{2+}$ = 611.49.

5.3 Pep$_3$ (NGYHN)

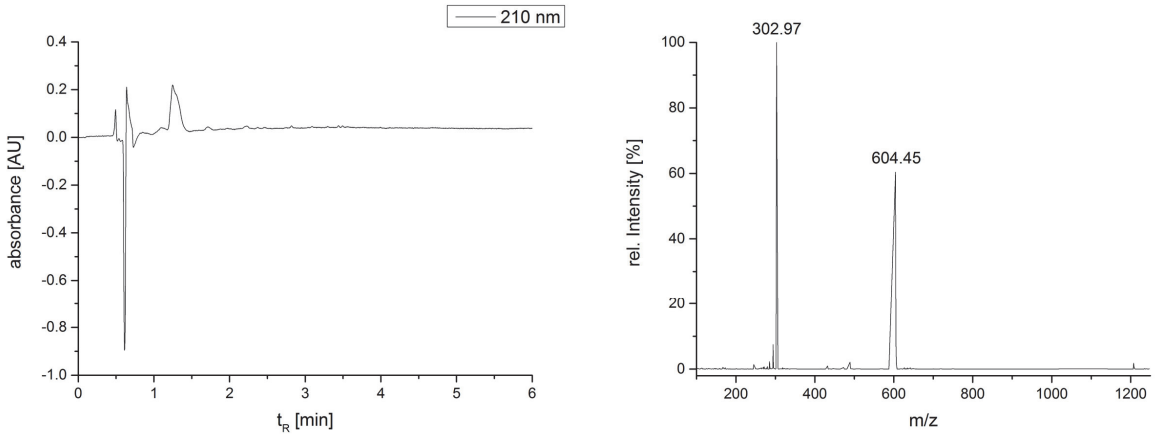


Figure S3. UPLC-MS analysis of Pep$_3$ (NGYHN), left: UV chromatogram at 210 nm, right: ESI-MS spectrum.

UV/VIS: t_R = 1.25 min. 93 % purity.

ESI-QMS: m/z calculated (average): $[M+H]^+$ = 603.62. $[M+2H]^{2+}$ = 302.21; found: $[M+H]^+$ = 604.5. $[M+2H]^{2+}$ = 303.0.

5.4 Pep$_4$ (NGYSS)

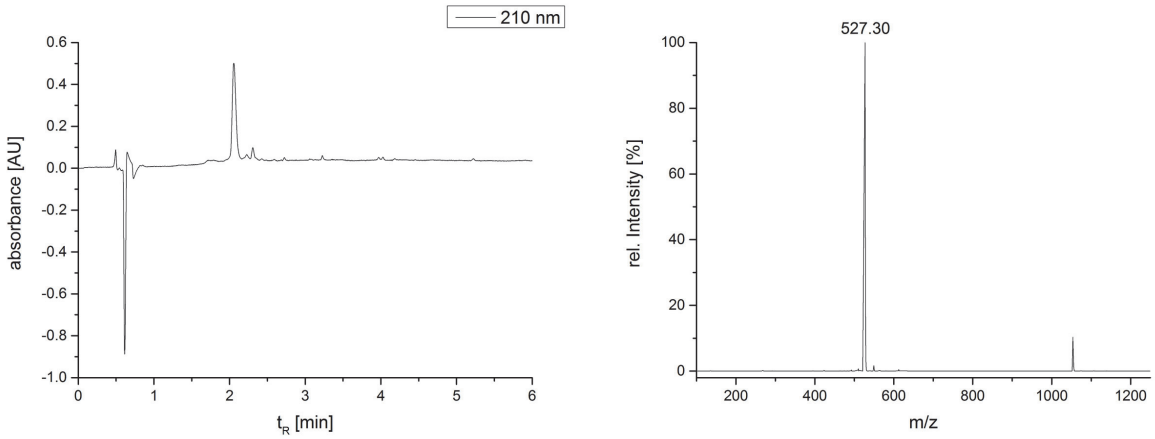


Figure S4. UPLC-MS analysis of Pep$_4$ (NGYSS), left: UV chromatogram at 210 nm, right: ESI-MS spectrum.

UV/VIS: t_R = 2.06 min. 86 % purity.

ESI-QMS: m/z calculated (average): $[M+H]^+$ = 526.53; found: $[M+H]^+$ = 527.3.

5.5 Pep$_5$ (SYYKS)

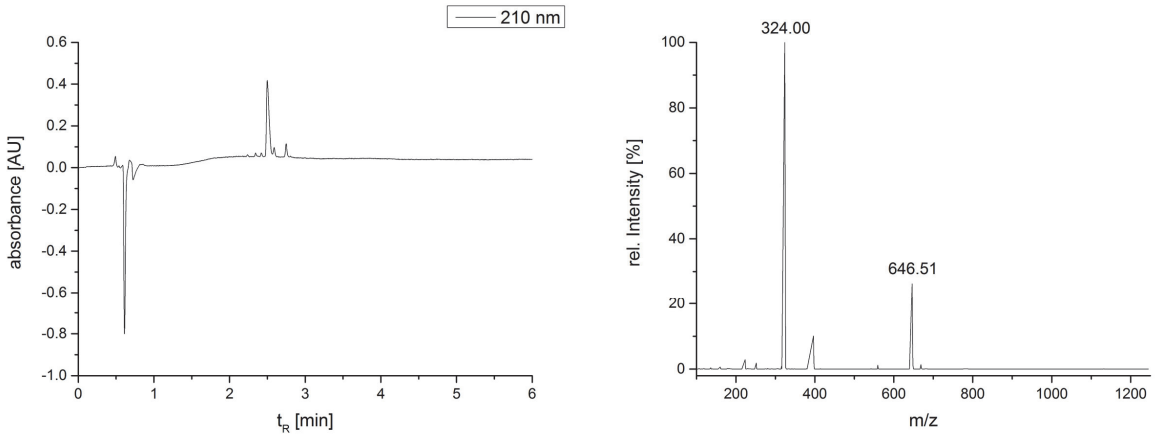


Figure S5. UPLC-MS analysis of Pep$_5$ (SYYKS), left: UV chromatogram at 210 nm, right: ESI-MS spectrum.

UV/VIS: t_R = 2.50 min. 80 % purity.

ESI-QMS: m/z calculated (average): $[M+H]^+$ = 646.72. $[M+2H]^{2+}$ = 323.86; found: $[M+H]^+$ = 646.5. $[M+2H]^{2+}$ = 324.0.

5.6 Pep$_6$ (SYYHP)

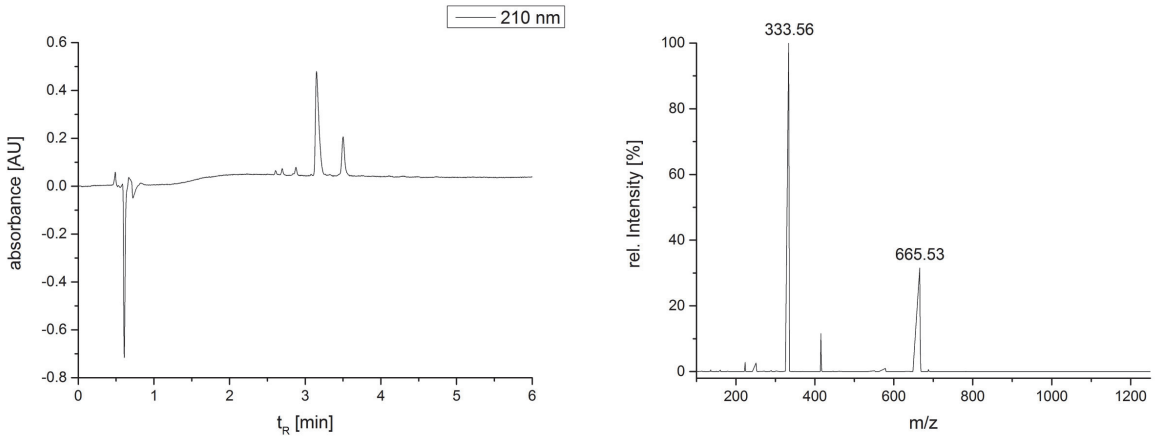


Figure S6. UPLC-MS analysis of Pep$_6$ (SYYHP), left: UV chromatogram at 210 nm, right: ESI-MS spectrum, t_R = 3.15 min.

UV/VIS: t_R = 3.15 min and 3.50 min 93 % purity.

ESI-QMS: t_R = 3.15 min; m/z calculated (average): $[M+H]^+$ = 665.73. $[M+2H]^{2+}$ = 333.37; found: $[M+H]^+$ = 665.5. $[M+2H]^{2+}$ = 333.6.

t_R = 3.50 min; m/z calculated (average): $[2M+Na+H]^{2+}$ = 676.72. found: $[2M+Na+H]^{2+}$ = 676.8.

5.7 Pep$_7$ (SPYAS)

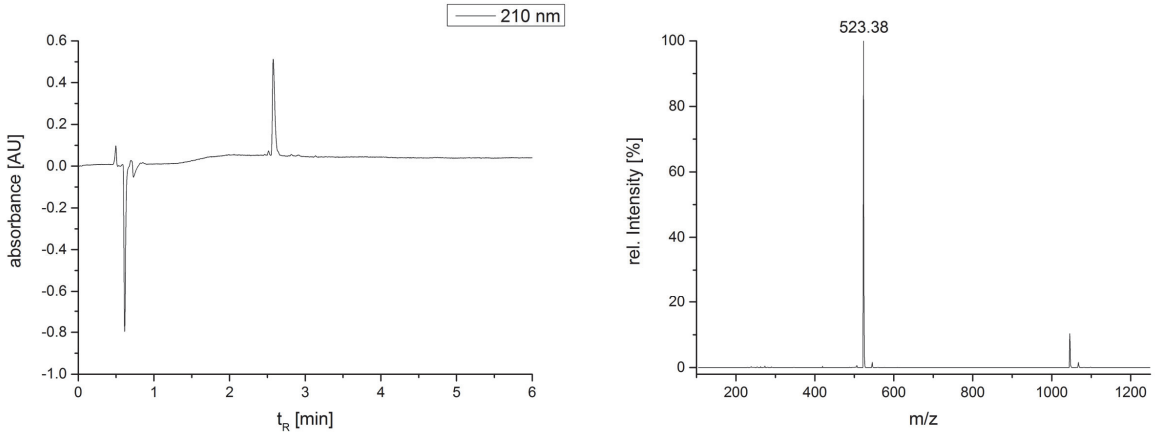


Figure S7. UPLC-MS analysis of Pep$_7$ (SPYAS), left: UV chromatogram at 210 nm, right: ESI-MS spectrum.

UV/VIS: t_R = 2.58 min. 96 % purity.

ESI-QMS: m/z calculated (average): $[M+H]^+$ = 523.57; found: $[M+H]^+$ = 523.4.

5.8 Pep$_8$ (NSYHS)

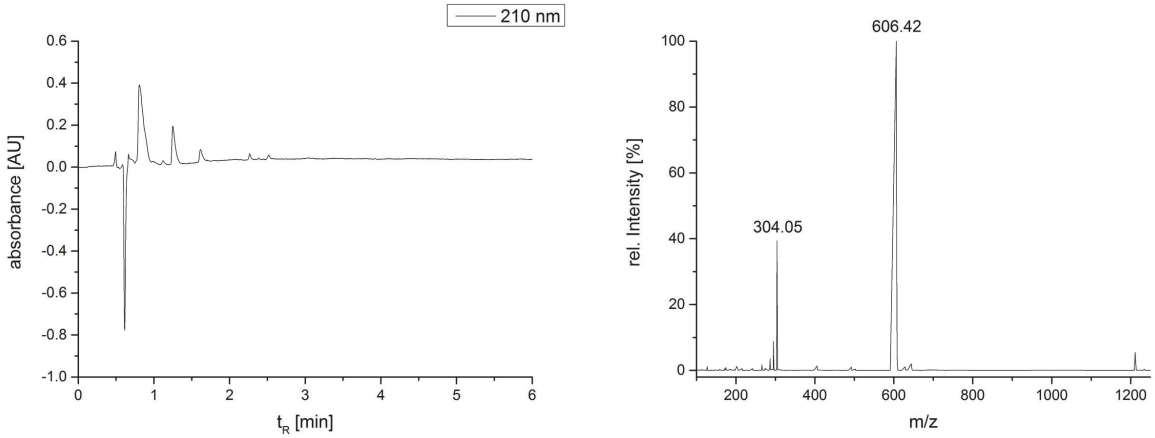


Figure S8. UPLC-MS analysis of Pep$_8$ (NSYHS), left: UV chromatogram at 210 nm, right: ESI-MS spectrum, t_R = 0.81 min.

UV/VIS: t_R = 0.81 min and 1.62 min. 76 % purity.

ESI-QMS: t_R = 0.81 min; m/z calculated (average): $[M+H]^+$ = 606.62. $[M+2H]^{2+}$ = 303.81; found: $[M+H]^+$ = 606.4. $[M+2H]^{2+}$ = 304.1.

t_R = 1.62 min; m/z calculated (average): $[2M+Na+H]^{2+}$ = 617.61. found: $[2M+Na+H]^{2+}$ = 617.7.

5.9 Pep$_9$ (KYYHP)

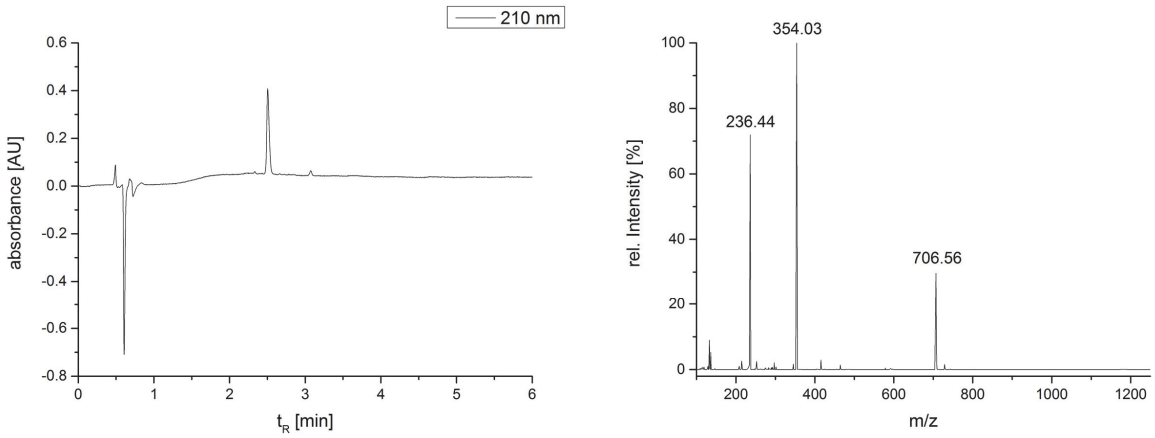


Figure S9. UPLC-MS analysis of Pep$_9$ (KYYHP), left: UV chromatogram at 210 nm, right: ESI-MS spectrum.

UV/VIS: t_R = 2.50 min. 94 % purity.

ESI-QMS: m/z calculated (average): $[M+H]^+$ = 706.82. $[M+2H]^{2+}$ = 353.91. $[M+3H]^{3+}$ = 236.28; found: $[M+H]^+$ = 706.6. $[M+2H]^{2+}$ = 354.0. $[M+3H]^{3+}$ = 236.4.

5.10 Pep$_{10}$ (PRYPR)

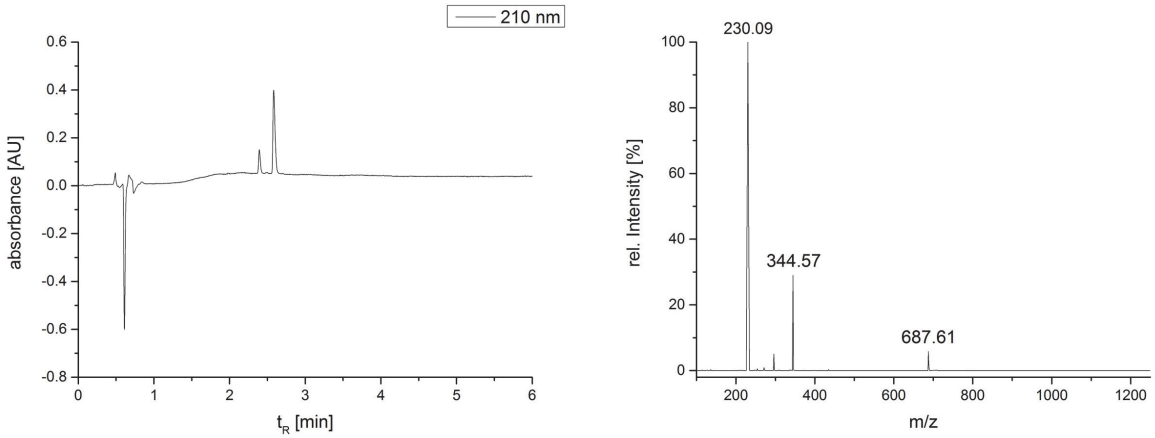


Figure S10. UPLC-MS analysis of Pep$_{10}$ (PRYPR), left: UV chromatogram at 210 nm, right: ESI-MS spectrum, t_R = 2.39 min.

UV/VIS: t_R = 2.39 min and 2.58 min. 99 % purity.

ESI-QMS: t_R = 2.39 min; m/z calculated (average): $[M+H]^+$ = 687.82. $[M+2H]^{2+}$ = 344.42. $[M+3H]^{3+}$ = 229.95; found: $[M+H]^+$ = 687.6. $[M+2H]^{2+}$ = 344.6. $[M+3H]^{3+}$ = 230.1.

t_R = 2.58 min; m/z calculated (average): $[2M+Na+H]^{2+}$ = 698.82; found: $[2M+Na+H]^{2+}$ = 699.7.

5.11 Pep$_{11}$ (AAYAP)

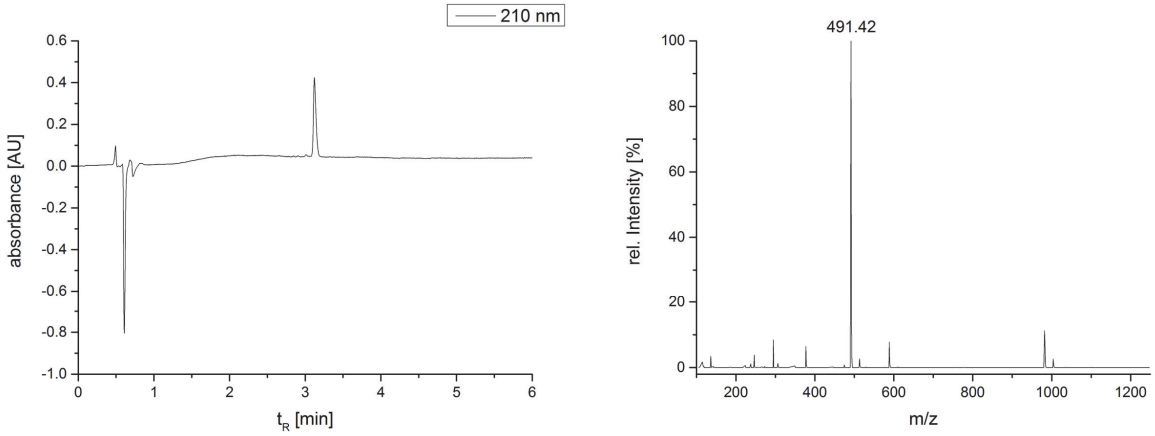


Figure S11. UPLC-MS analysis of Pep$_{11}$ (AAYAP), left: UV chromatogram at 210 nm, right: ESI-MS spectrum.

UV/VIS: t_R = 3.12 min. 98 % purity.

ESI-QMS: m/z calculated (average): $[M+H]^+$ = 491.57; found: $[M+H]^+$ = 491.4.

5.12 peptide-PEG conjugate $(Pep_2)_3$-PEG_{108}

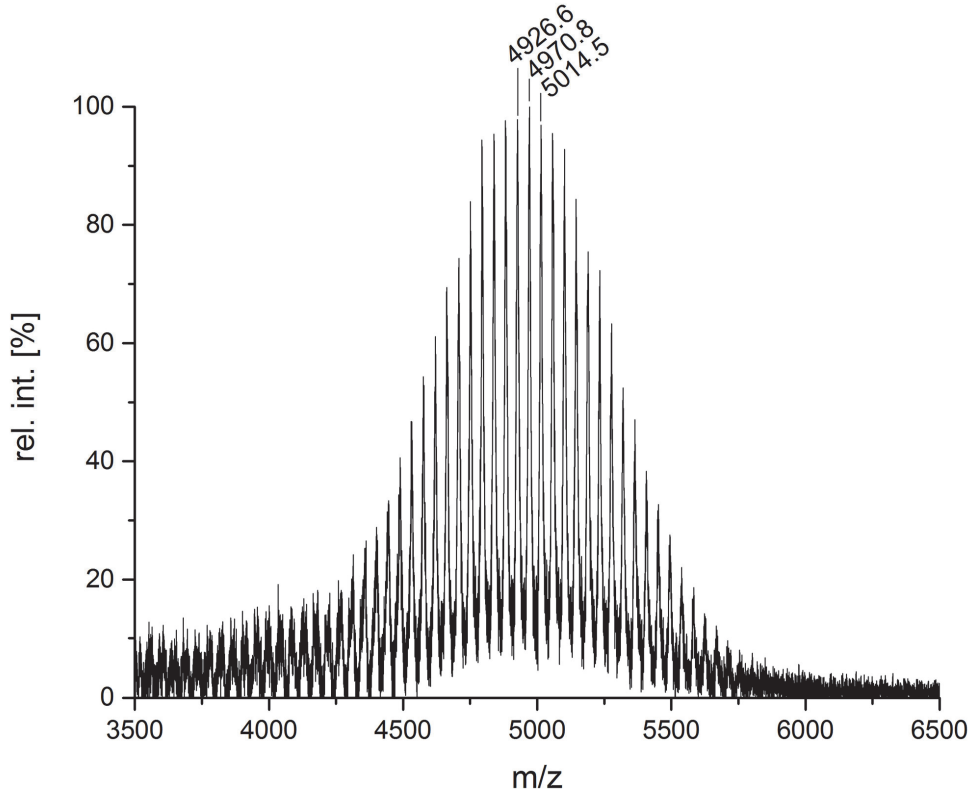


Figure S12. MALDI-TOF-MS analysis of $(Pep_2)_3$-PEG_{108}. The spectrum is baseline corrected.

Δm(PEG) = 44.05 Da (average). characteristic of the EG repeat units.

M(pep) = 1761.8 Da $(HSYHG)_3$ (average).

M(peak) = m/z 4971 assignable to $[M(pep)+PEG_{72}+K]^+$ = 4972 Da within ± 1 Da accuracy.

^{1}H-NMR (500 MHz. TFA-d. δ in ppm): 8.63-8.44 (m. 6H. $CH_{ar.H}$). 7.54-7.25 (m. 6H. $CH_{ar.H}$). 7.08-6.99 (m. 6H. $CH_{ar.Y}$). 6.85-6.75 (m. 6H. $CH_{ar.Y}$). 5.11-5.02 (m. 3H. α-CH_Y). 5.00-4.53 (m. 9H. α-CH_H. α-CH_S). 4.27-3.95 (m. 12H. α-$CH_{2.G}$. β-$CH_{2.S}$). 3.94-3.78 (m. 432H. $CH_{2.PEG}$). 3.73-3.68 (m. 2H. CH_2). 3.63-3.56 (m. 2H. CH_2). 3.47-3.11 (m. 12H. β-$CH_{2.H}$). 3.10-2.91 (m. 6H. β-$CH_{2.Y}$).

5.13 peptide-PEG conjugate $(Pep_4)_3$-PEG_{81}

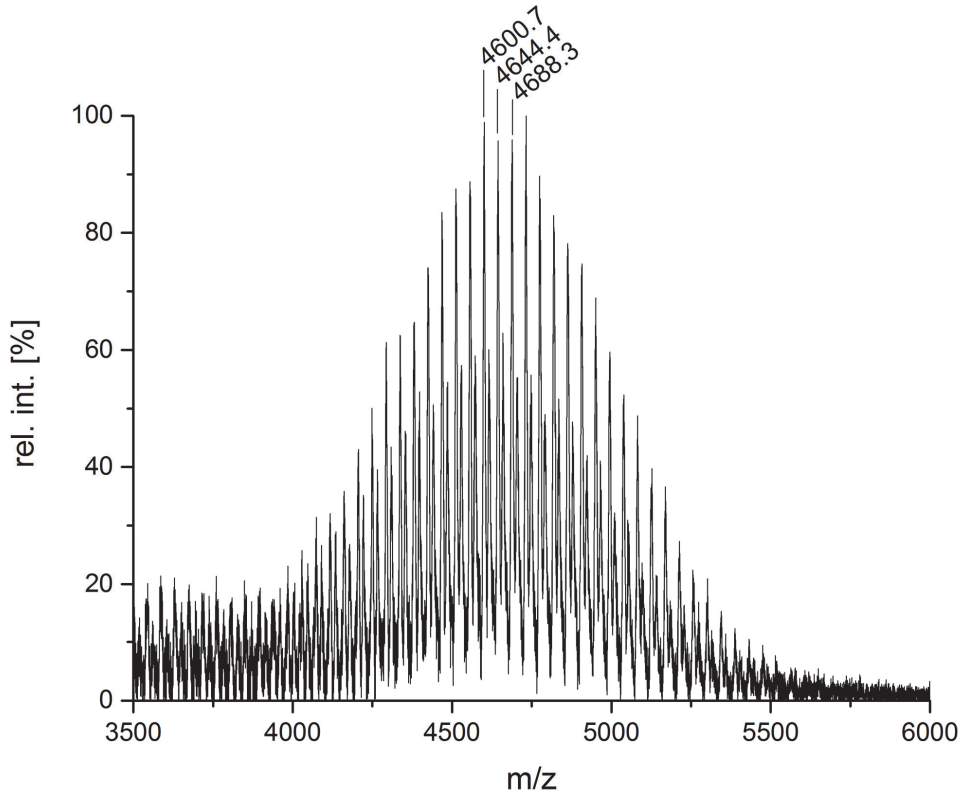


Figure S13. MALDI-TOF-MS analysis of $(Pep_4)_3$-PEG_{81}. The spectrum is baseline corrected.

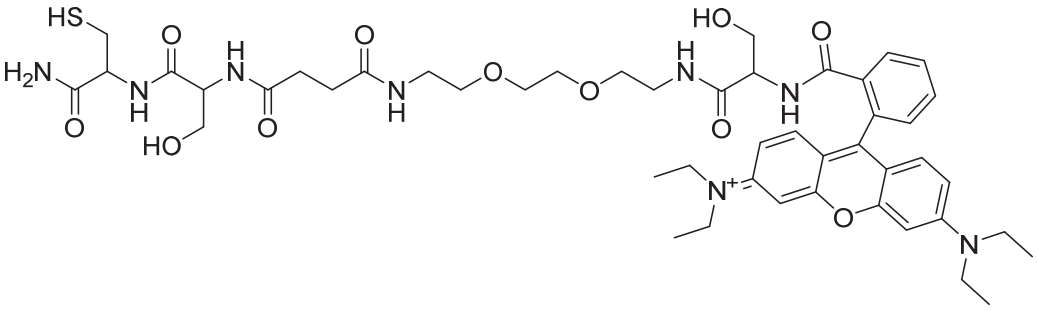
Δm(PEG) = 44.03 Da (exact). characteristic of the EG repeat units.

M(pep) = 1541.6 Da $(NGYSS)_3$ (exact).

M(peak) = m/z 4644.4 assignable to $[M(pep)+PEG_{70}+Na]^+$ = 4645.6 Da.

^{1}H-NMR (500 MHz. TFA-d. δ in ppm): 7.15-7.03 (m. 6H. $CH_{ar.Y}$). 6.91-6.80 (m. 6H. $CH_{ar.Y}$). 5.17-5.06 (m. 3H. α-CH_Y). 4.94-4.56 (m. 9H. α-CH_N. α-CH_S). 4.33-3.96 (m. 18H. α-$CH_{2.G}$. β-$CH_{2.S}$). 3.95-3.76 (m. 324H. $CH_{2.PEG}$). 3.75-3.69 (m. 2H. CH_2). 3.64-3.58 (m. 2H. CH_2). 3.29-2.93 (m. 12H. β-$CH_{2.N}$. β-$CH_{2.Y}$).

5.14 Fluorescent probe



MALDI-TOF-MS

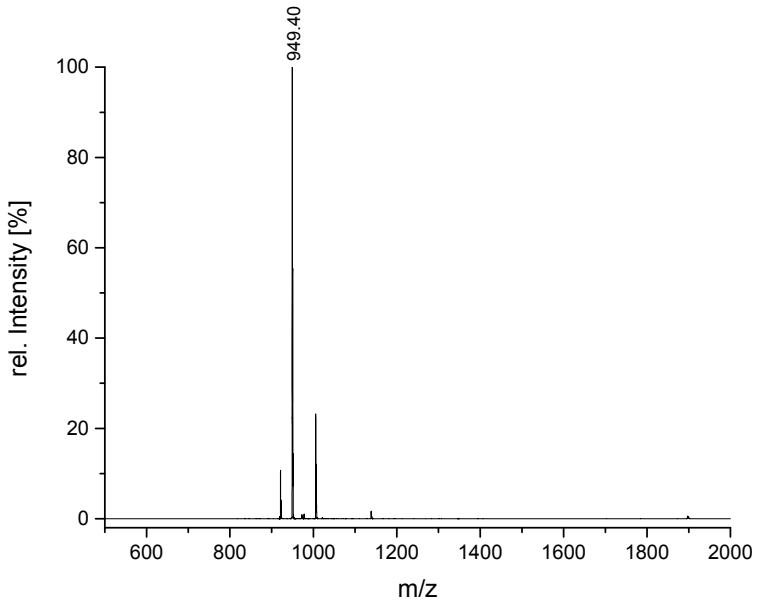


Figure S14. MALDI-TOF-MS analysis of the fluorescent probe.

m/z calc. (monoisotopic): $[M]^+ = 949.7$; found: $[M]^+ = 949.4$.

UPLC-UV/VIS-QMS

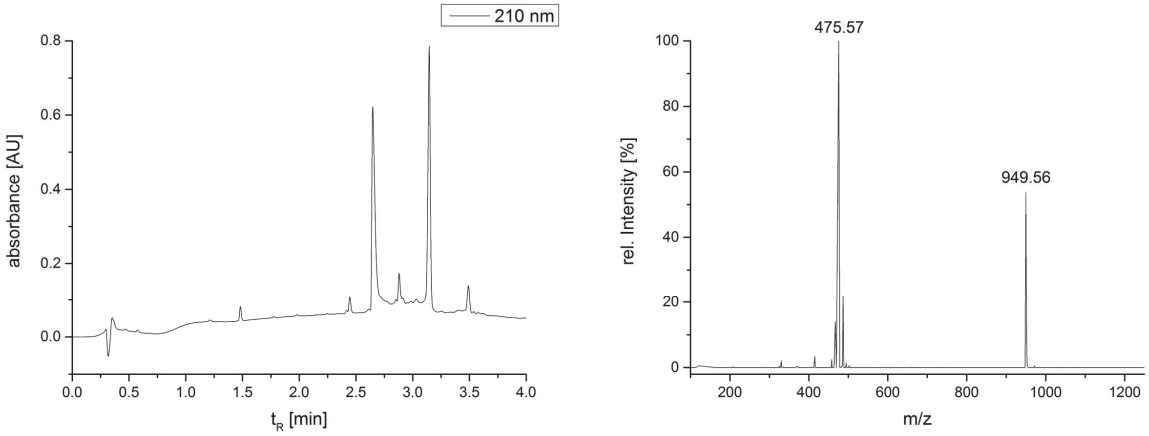


Figure S15. UPLC-MS analysis of the fluorescent probe, left: UV chromatogram at 210 nm, right: ESI-MS spectrum.

UV/VIS: t_R = 2.65 and 3.15 min. 91% purity.

ESI-QMS: m/z calculated (average): $[M+H]^+$ = 950.14. $[M+2H]^{2+}$ = 475.57; found: $[M+H]^+$ = 949.6. $[M+H]^{2+}$ = 475.6.

6. Experiments

6.1 UV kinetic measurements of peptides Pep$_1$ - Pep$_{11}$

Peptides were activated according to protocol 3.8. Absorbance of peptides at 305 nm was followed over time.

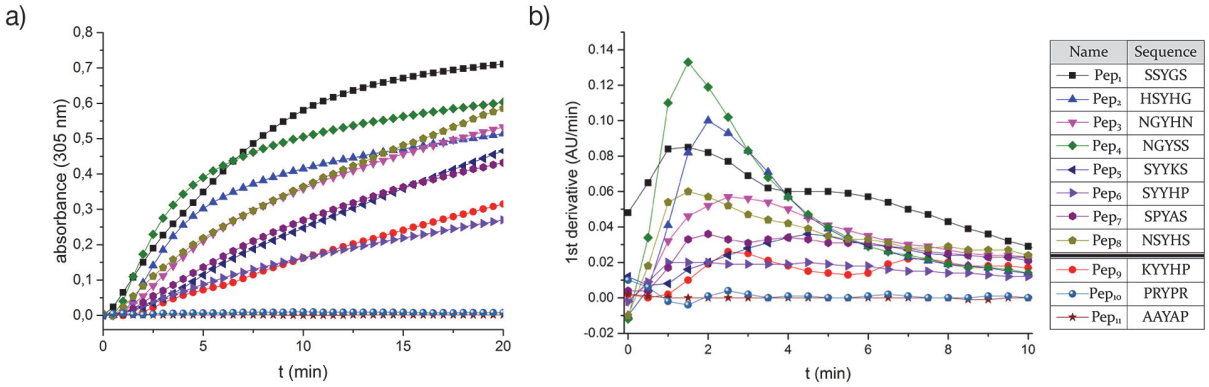


Figure S16. Kinetic activation experiments of peptides Pep$_1$ – Pep$_{11}$. a) UV absorbance at 305 nm. b) The first deviation of the UV Signal reveals that Pep$_2$ and Pep$_4$ have the highest maximum activation rate.

Table S1. Maximum activation rates for Pep$_{1\text{-}11}$ from the first derivative of kinetic UV/Vis measurements.

peptide	**maximum activation rate [AU/min]**
Pep$_1$	0.085
Pep$_2$	0.1
Pep$_3$	0.057
Pep$_4$	0.133
Pep$_5$	0.036
Pep$_6$	0.02
Pep$_7$	0.036
Pep$_8$	0.06
Pep$_9$	0.026
Pep$_{10}$	0.01
Pep$_{11}$	0.002

6.2 UV kinetic measurements of peptide-PEG conjugate activation

0.25 µmol/mL of conjugates $(Pep_2)_3$-PEG and $(Pep_4)_3$-PEG were each activated to Dopaquinone according to protocol 3.8 and UV absorbance at 305 nm was measured over time. Final absorbance of 1.5 AU is reached after 120 min for $(Pep_4)_3$-PEG and 1.1 AU is reached after 60 min for $(Pep_2)_3$-PEG.

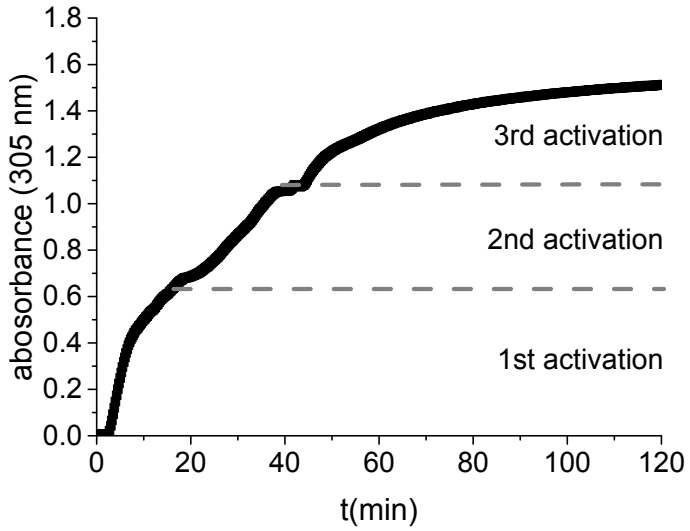


Figure S17. Kinetic UV absorbance measurement of peptide-PEG conjugate $(Pep_4)_3$-PEG.

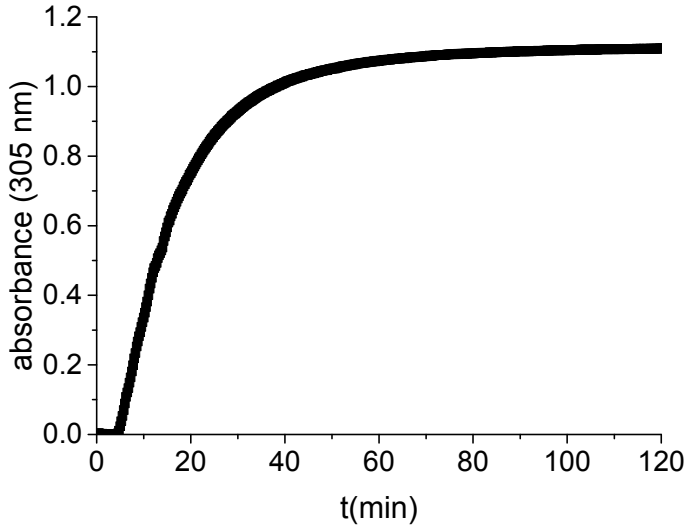


Figure S18. Kinetic UV absorbance measurement of peptide-PEG conjugate $(Pep_2)_3$-PEG.

6.3 MALDI-TOF-MS measurements of activated peptide-PEG conjugates

Conjugates $(Pep_2)_3$-PEG and $(Pep_4)_3$-PEG were each activated to Dopa for 1.5 h according to protocol 3.8 using a concentration 0.25 µmol/mL.

6.3.1 Activation experiment of conjugate $(Pep_2)_3$-PEG

The mass signal of native conjugate (A) is visible along with masses of one oxidized tyrosine residue (B) and two oxidized tyrosine residues (C) to Dopa each shifted by +16 Da.

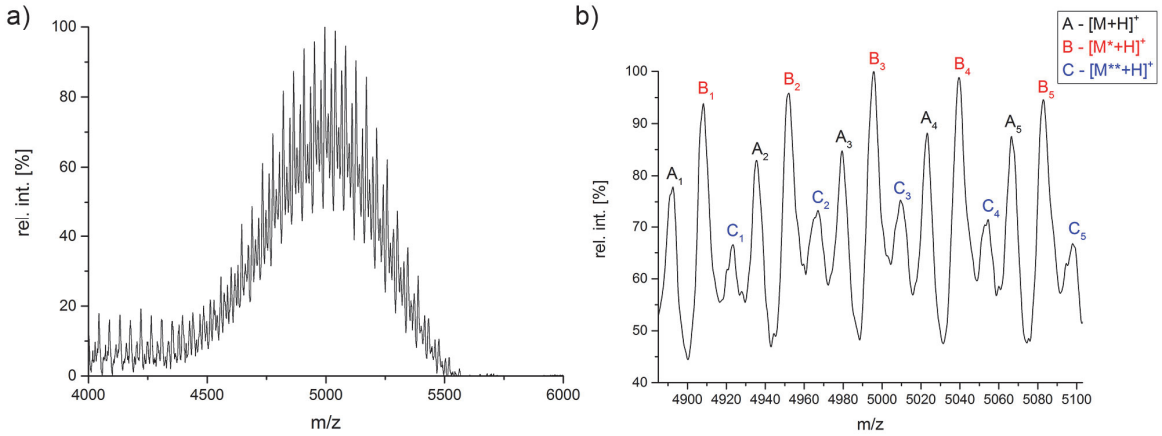


Figure S19. MALDI-TOF-MS spectrum of activated conjugate $(Pep_2)_3$-PEG. a) full spectrum. b) close-up view showing the different assigned species. (*one oxidized residue. **two oxidized residues).

Masses of the different species could be assigned with an average deviation of 0.9 Da (cf. Table S2). Average shifts for species B = 16.4 Da and for species C = 31.5 Da can be assigned to one- and two-fold oxidized (Dopa) conjugate.

Δm(PEG) = 44.05 Da (average). characteristic of the EG repeat units.

M(pep) = 1761.8 Da $(HSYHG)_3$ (average).

Table S2. Peak list of assigned species of activated conjugate $(Pep_2)_3$-PEG.

assigned species	m/z	calculated	Δ
A_1	4891.63	[M(pep)+PEG_{71}+1(H^+)] 4890.35	-1.28
B_1	4908.25	[M(pep)+PEG_{71}+16(1xDopa)+1(H^+)] 4906.35	-1.9
C_1	4923.22	[M(pep)+PEG_{71}+32(2xDopa)+1(H^+)] 4922.35	-0.87
A_2	4935.86	[M(pep)+PEG_{72}+1(H^+)] 4934.4	-1.46
B_2	4952.13	[M(pep)+PEG_{72}+16(1xDopa)+1(H^+)] 4950.4	-1.73
C_2	4967.1	[M(pep)+PEG_{72}+32(2xDopa)+1(H^+)] 4966.4	-0.7
A_3	4979.46	[M(pep)+PEG_{73}+1(H^+)] 4978.45	-1.01
B_3	4995.56	[M(pep)+PEG_{73}+16(1xDopa)+1(H^+)] 4994.45	-1.11
C_3	5010.94	[M(pep)+PEG_{73}+32(2xDopa)+1(H^+)] 5010.45	-0.49
A_4	5023.01	[M(pep)+PEG_{74}+1(H^+)] 5022.5	-0.51
B_4	5039.55	[M(pep)+PEG_{74}+16(1xDopa)+1(H^+)] 5038.5	-1.05
C_4	5054.12	[M(pep)+PEG_{74}+32(2xDopa)+1(H^+)] 5054.5	0.38
A_5	5066.59	[M(pep)+PEG_{75}+1(H^+)] 5066.55	-0.04
B_5	5083.23	[M(pep)+PEG_{75}+16(1xDopa)+1(H^+)] 5082.55	-0.68
C_5	5098.49	[M(pep)+PEG_{75}+32(2xDopa)+1(H^+)] 5098.55	0.06

6.3.2 Activation experiment of conjugate $(Pep_4)_3$-PEG

The mass signals of conjugate with one oxidized tyrosine residue (A). two oxidized tyrosine residues (B) and three oxidized tyrosine residues (C) are visible. No mass signal for native conjugate is observed.

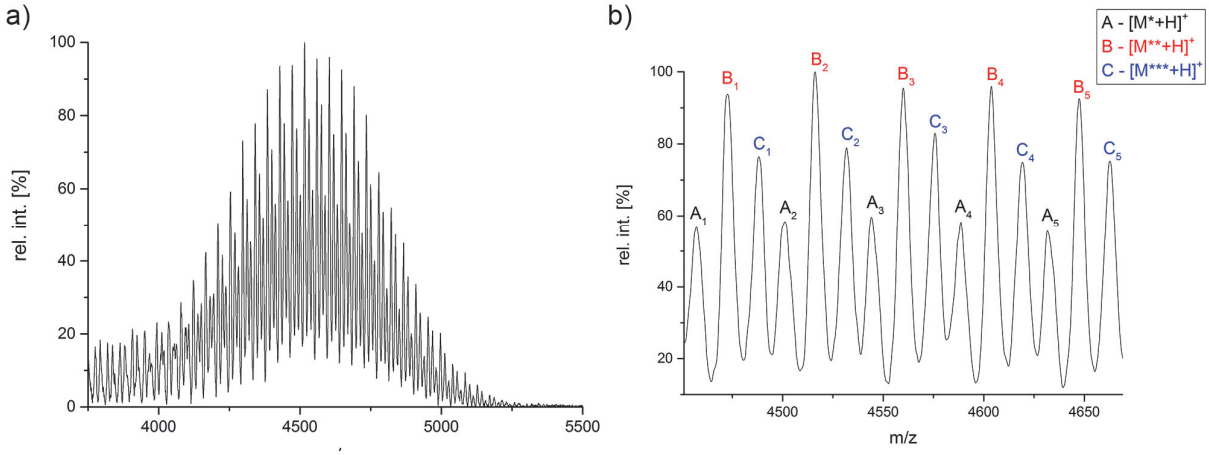


Figure S20. MALDI-TOF-MS spectrum of activated conjugate $(Pep_4)_3$-PEG. a) full spectrum. b) close-up view showing the different assigned species. (*one oxidized residue. **two oxidized residues. ***three oxidized residues).

Masses of the different species could be assigned with an average deviation of 2.6 Da (cf. Table S3). Average shifts for species B = 15.4 Da and for species C = 15.4 Da with respect to the previous peak can be assigned to two- and three-fold oxidized (Dopa) conjugate.

Δm(PEG) = 44.03 Da (exact). characteristic of the EG repeat units.

M(pep) = 1541.6 Da $(NGYSS)_3$ (exact).

Table S3. Peaklist of assigned species of activated conjugate $(Pep_4)_3$-PEG and comparison to calculated masses.

assigned species	m/z	calculated	Δ
A_1	4457.29	[M(pep)+PEG_{65}+16(1xDopa)+39.1(K^+)] 4458.65	1.36
B_1	4472.72	[M(pep)+PEG_{65}+32(2xDopa)+39.1(K^+)] 4474.65	1.93
C_1	4488.14	[M(pep)+PEG_{65}+48(3xDopa)+39.1(K^+)] 4490.65	2.51
A_2	4500.92	[M(pep)+PEG_{66}+16(1xDopa)+39.1(K^+)] 4502.68	1.76
B_2	4516.3	[M(pep)+PEG_{66}+32(2xDopa)+39.1(K^+)] 4518.68	2.38
C_2	4531.72	[M(pep)+PEG_{66}+48(3xDopa)+39.1(K^+)] 4534.68	2.96
A_3	4544.58	[M(pep)+PEG_{67}+16(1xDopa)+39.1(K^+)] 4546.71	2.13
B_3	4560.11	[M(pep)+PEG_{67}+32(2xDopa)+39.1(K^+)] 4562.71	2.6
C_3	4575.47	[M(pep)+PEG_{67}+48(3xDopa)+39.1(K^+)] 4578.71	3.24
A_4	4588.36	[M(pep)+PEG_{68}+16(1xDopa)+39.1(K^+)] 4590.74	2.38
B_4	4603.73	[M(pep)+PEG_{68}+32(2xDopa)+39.1(K^+)] 4606.74	3.01
C_4	4619.24	[M(pep)+PEG_{68}+48(3xDopa)+39.1(K^+)] 4622.74	3.5
A_5	4632.44	[M(pep)+PEG_{69}+16(1xDopa)+39.1(K^+)] 4634.77	2.33
B_5	4647.51	[M(pep)+PEG_{69}+32(2xDopa)+39.1(K^+)] 4650.77	3.26
C_5	4662.79	[M(pep)+PEG_{69}+48(3xDopa)+39.1(K^+)] 4666.77	3.98

6.4 QCM adsorption desorption experiments

For QCM measurements sample concentration (0.25 µmol/mL) had to be reduced and samples were diluted after reaction prior to measurement with Milli-Q water 1:21 v/v (0.01 µmol/mL). Therefore potassium phosphate buffer (17 mM. pH 6.5) was diluted to 0.8 mM as well for use in equilibration and rinsing steps. All measurements were performed at a flow of 100 µL/min according to protocol 3.9.

6.4.1 Tyrosinase reference experiment

This experiment was conducted to exclude any adsorption observed for other measurements comes from tyrosinase. A solution of 100 U/mL tyrosinase and 6.9 mmol/mL of sodium ascorbate was prepared and diluted with Milli-Q water as described above. Incubation of the enzyme over a period of 1.5 h showed no significant adsorption.

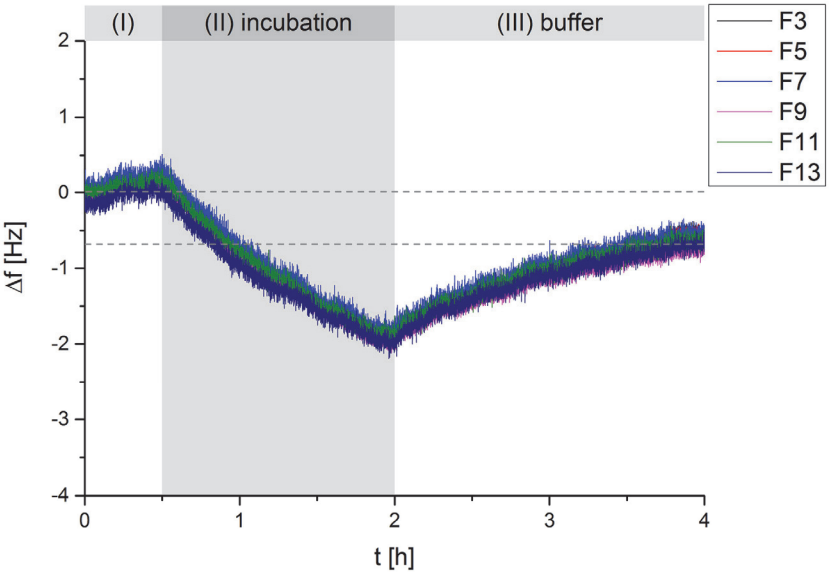


Figure S21. QCM adsorption and desorption kinetics of tyrosinase on Al_2O_3 showing frequency overtones F3 – F13.

1. phosphate buffer solution (0.8 mM. pH 6.5)
2. tyrosinase solution (4.5 U/mL)
3. phosphate buffer solution (0.8 mM. pH 6.5)

6.4.2 peptide-PEG conjugate $(Pep_2)_3$-PEG control

As a control experiment QCM measurement of native conjugate solution was carried out. A conjugate layer adsorbed onto the alumina surface which was fully removed by rinsing with 599 mM NaCl solution serving as a sea water model solution.

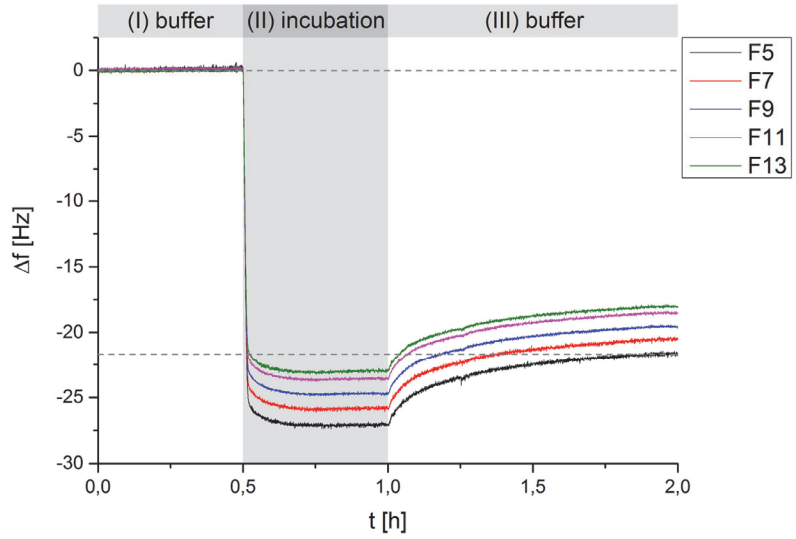


Figure S22. QCM adsorption and desorption kinetics of $(Pep_2)_3$-PEG surface modification on Al_2O_3 showing frequency overtones F5 – F13.

I. phosphate buffer solution (0.8 mM. pH 6.5)
II. $(Pep_2)_3$-PEG solution (0.01 µM in phosphate buffer)
III. phosphate buffer solution (0.8 mM. pH 6.5)

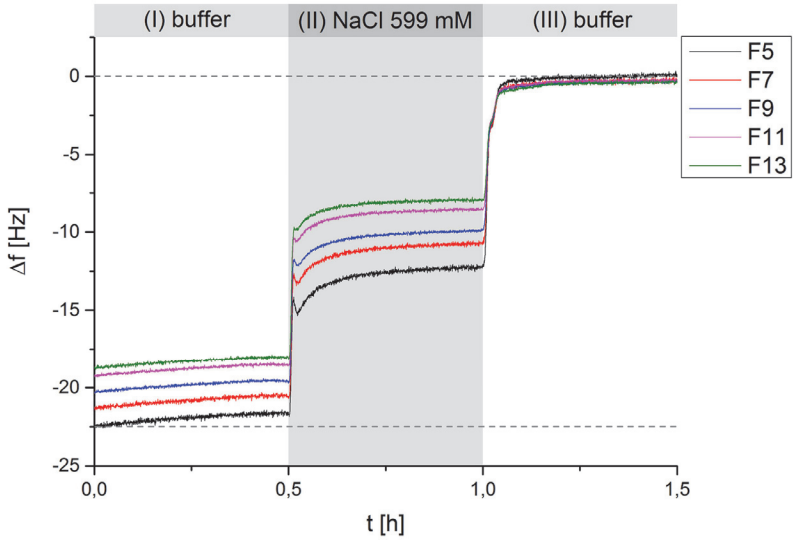


Figure S23. QCM adsorption and desorption kinetics of rinsing $(Pep_2)_3$-PEG modified Al_2O_3 surface with NaCl solution (599 mM) showing frequency overtones F5 – F13.

I. phosphate buffer solution (0.8 mM. pH 6.5)
II. NaCl solution (599 mM)
III. phosphate buffer solution (0.8 mM. pH 6.5)

6.4.3 activated peptide-PEG conjugate $(Pep_2^*)_3$-PEG_{3000}

Conjugate was activated to catechol for 1.5 h according to protocol 3.8. then diluted and used for QCM measurement. Part of the adsorbed layer resisted washing off with NaCl solution showing increased adhesive properties of activated conjugate compared to native conjugate.

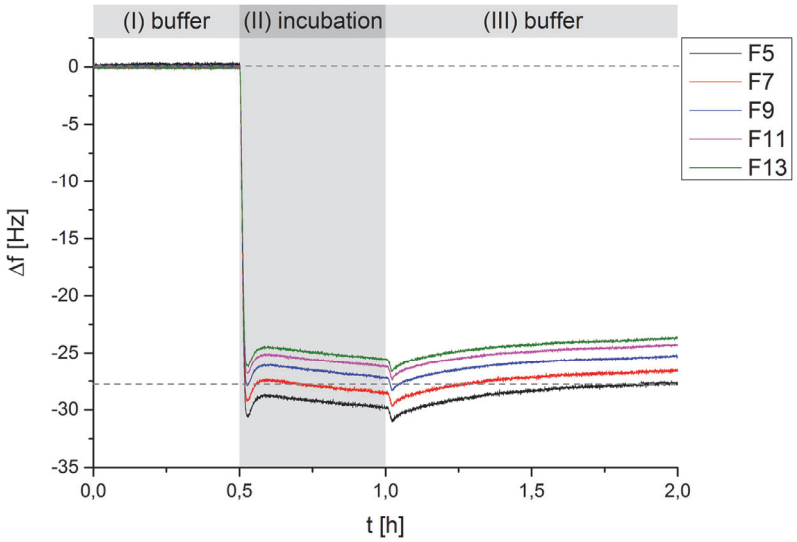


Figure S24. QCM adsorption and desorption kinetics of activated $(Pep_2^*)_3$-PEG_{3000} surface modification on Al_2O_3 showing frequency overtones F5 – F13.

I. phosphate buffer solution (0.8 mM. pH 6.5)
II. $(Pep_2^*)_3$-PEG_{3000} solution (0.01 µM in phosphate buffer)
III. phosphate buffer solution (0.8 mM. pH 6.5)

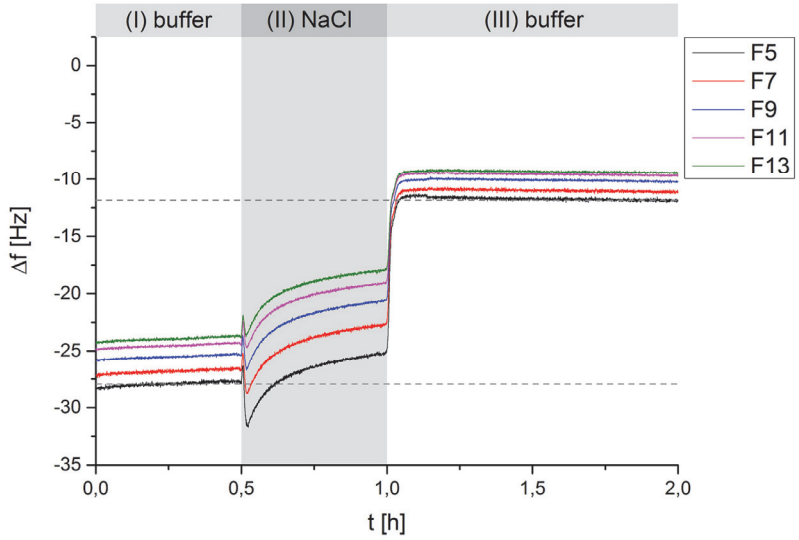


Figure S25. QCM adsorption and desorption kinetics of rinsing $(Pep_2^*)_3$-PEG_{3000} modified Al_2O_3 surface with NaCl solution (599 mM) showing frequency overtones F5 – F13.

I. phosphate buffer solution (0.8 mM. pH 6.5)
II. NaCl solution (599 mM)
III. phosphate buffer solution (0.8 mM. pH 6.5)

6.4.4 peptide-PEG conjugate $(Pep_4)_3$-PEG control

A control experiment with a solution of native conjugate was carried out. QCM measurement shows that no adsorption takes place.

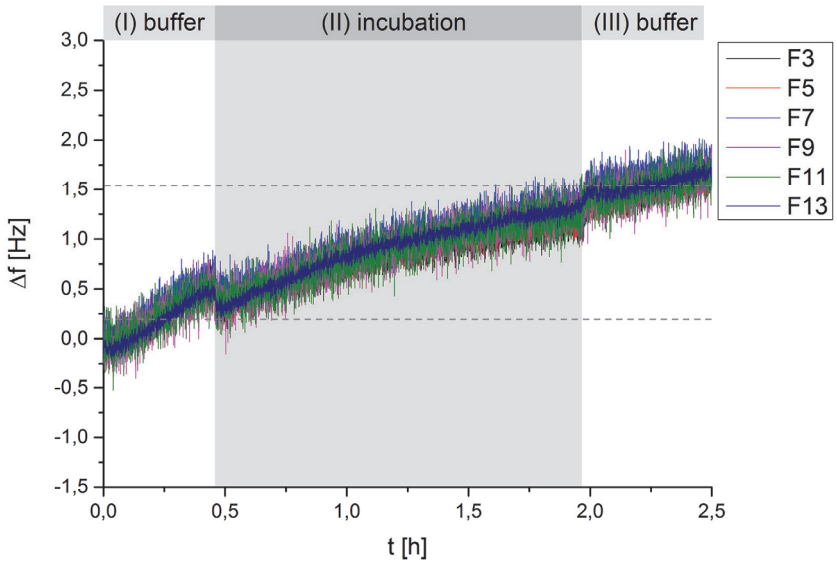


Figure S26. QCM adsorption and desorption kinetics of $(Pep_4)_3$-PEG surface modification on Al_2O_3 showing frequency overtones F3 – F13.

I. phosphate buffer solution (0.8 mM. pH 6.5)
II. $(Pep_4)_3$PEG solution (0.01 µM in phosphate buffer)
III. phosphate buffer solution (0.8 mM. pH 6.5)

6.4.5 activated peptide-PEG conjugate $(Pep_4*)_3$-PEG_{3000}

Conjugate was activated to catechol for 1.5 h according to protocol 3.8. then diluted and used for QCM measurement. During incubation step (II) an adsorbed layer is formed resulting in a minor frequency shift of $\Delta f = -10$ Hz. The layer is not stable and is slowly washed off the Al_2O_3 surface during buffer rinsing step (III).

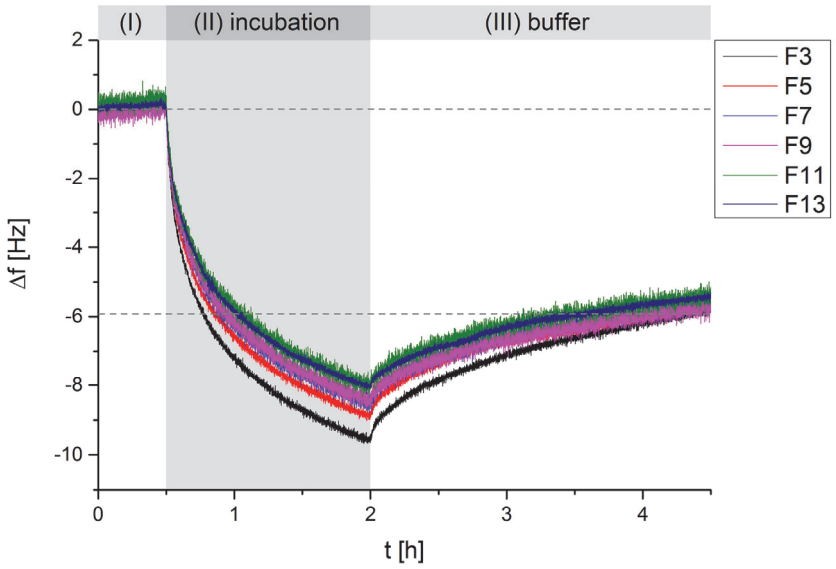


Figure S27. QCM adsorption and desorption kinetics of activated $(Pep_4*)_3$-PEG_{3000} surface modification on Al_2O_3 showing frequency overtones F3 – F13.

I. phosphate buffer solution (0.8 mM. pH 6.5)
II. $(Pep_4*)_3$-PEG_{3000} solution (0.01 µM in phosphate buffer)
III. phosphate buffer solution (0.8 mM. pH 6.5)

6.5 Fluorescence microscopic experiments

6.5.1 Autofluorescence of Fmoc Aminomethyl ChemMatrix resin

1.0 mg of Fmoc Aminomethyl ChemMatrix resin was swollen in Milli-Q water for more than one hour and afterwards examined under the fluorescence microscope. No autofluorescence was observed.

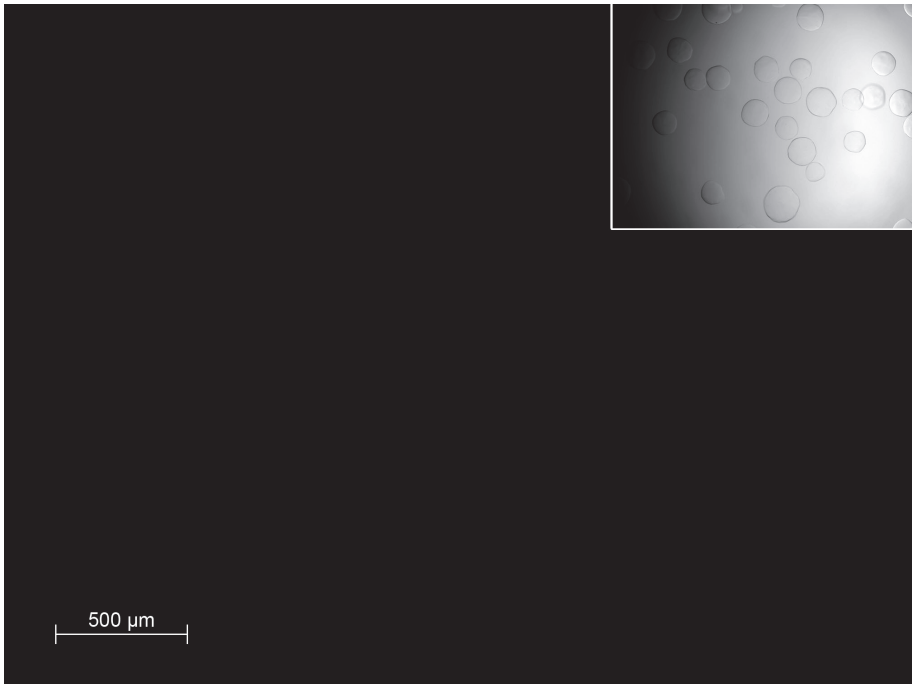


Figure S28. Fluorescence microscopic images of ChemMatrix resin (offset transmission mode).

6.5.2 Interaction of Fmoc Aminomethyl ChemMatrix resin with the fluorescent probe

1.2 mg of Fmoc Aminomethyl ChemMatrix resin (1.2 µmol) was swollen in Milli-Q water for more than one hour. The resin was incubated in a solution of 0.57 mg (0.60 µmol. 0.5 eq) fluorescent probe in 1 mL Milli-Q water for 5 min. Subsequently the resin was washed with MeOH (3x) and Milli-Q water (2x) and examined under the fluorescence microscope. An interaction of the fluorescent probe with ChemMatrix resin can be excluded. as no increased fluorescence was observed.

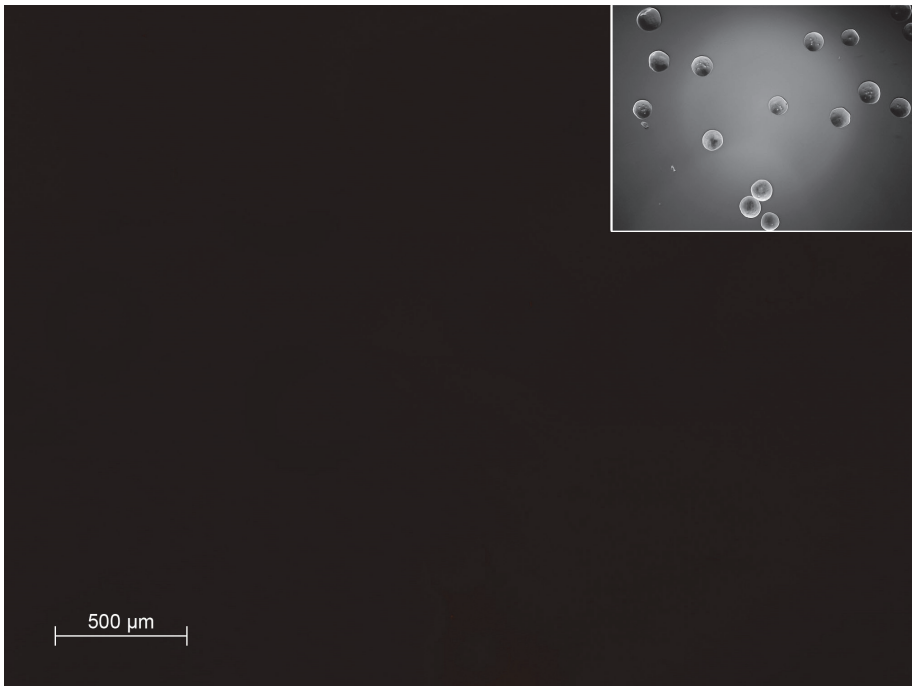


Figure S29. Fluorescence microscopic images of ChemMatrix resin after incubation with the fluorescent probe (offset transmission mode).

6.5.4 Autofluorescence of Testpep$_1$

1.3 mg of Testpep$_1$ resin (Ac-YGG-GGGM) was swollen in Milli-Q water for more than one hour and afterwards examined under the fluorescence microscope. No autofluorescence was observed.

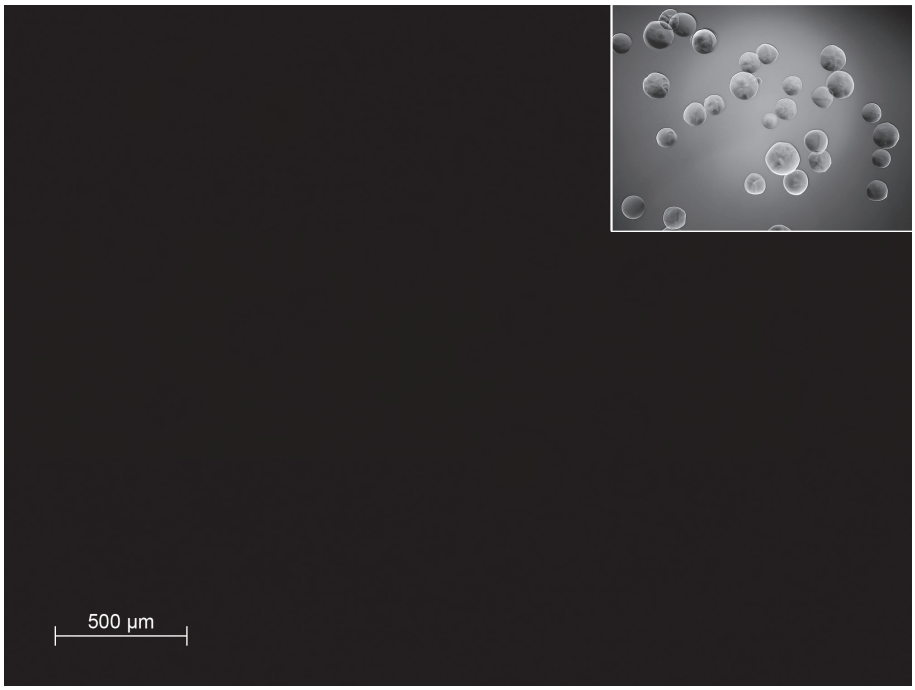


Figure S30. Fluorescence microscopic images of Testpep$_1$ resin (offset transmission mode).

6.5.4 Interaction of Testpep$_1$ with the fluorescent probe

1.0 mg of Testpep$_1$ resin (0.29 µmol. Ac-YGG-GGGM) was swollen in Milli-Q water for more than one hour. Afterwards the resin was incubated in a solution of 0.14 mg (0.15 µmol. 0.5 eq) fluorescent probe in 1 mL Milli-Q water for 5 min. Subsequently the resin was washed with MeOH (3x) and Milli-Q water (2x) and examined under the fluorescence microscope. No increased fluorescence was observed. therefore there were no interactions of the fluorescent probe with Testpep$_1$.

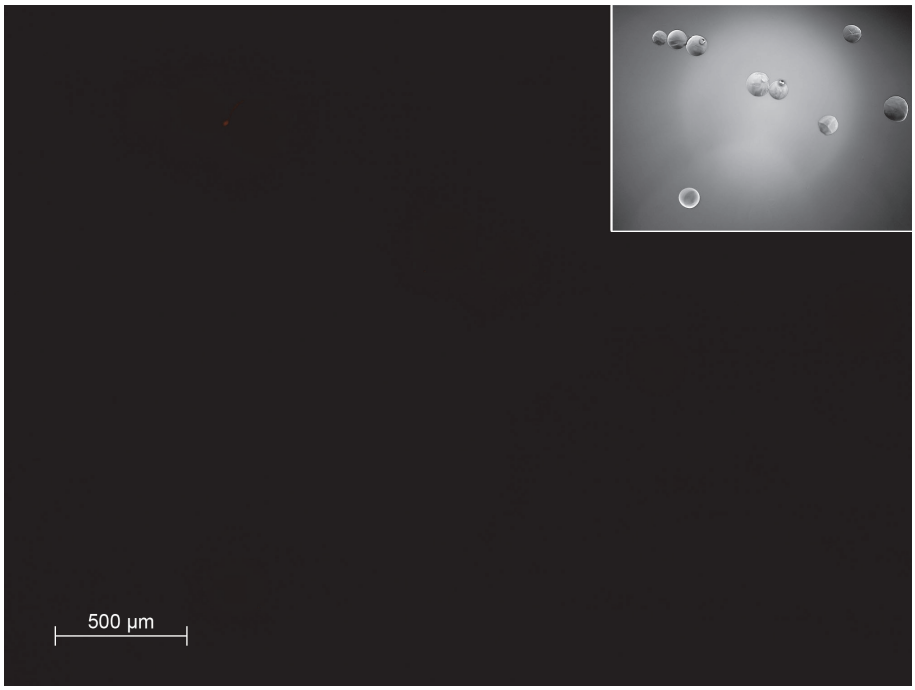


Figure S31. Fluorescence microscopic images of Testpep$_1$ resin after incubation with the fluorescent probe (offset transmission mode).

6.5.5 Activation and labeling of Testpep$_1$

1.1 mg of Testpep$_1$ resin (0.32 µmol. Ac-YGG-GGGM) was enzymatically activated and labeled according to protocol 3.7 using 0.15 mg (0.16 µmol. 0.5 eq) fluorescent probe. An increase in fluorescence intensity shows successful ligation of the dopaquinone moiety of the oxidized peptide with the fluorescent probe.

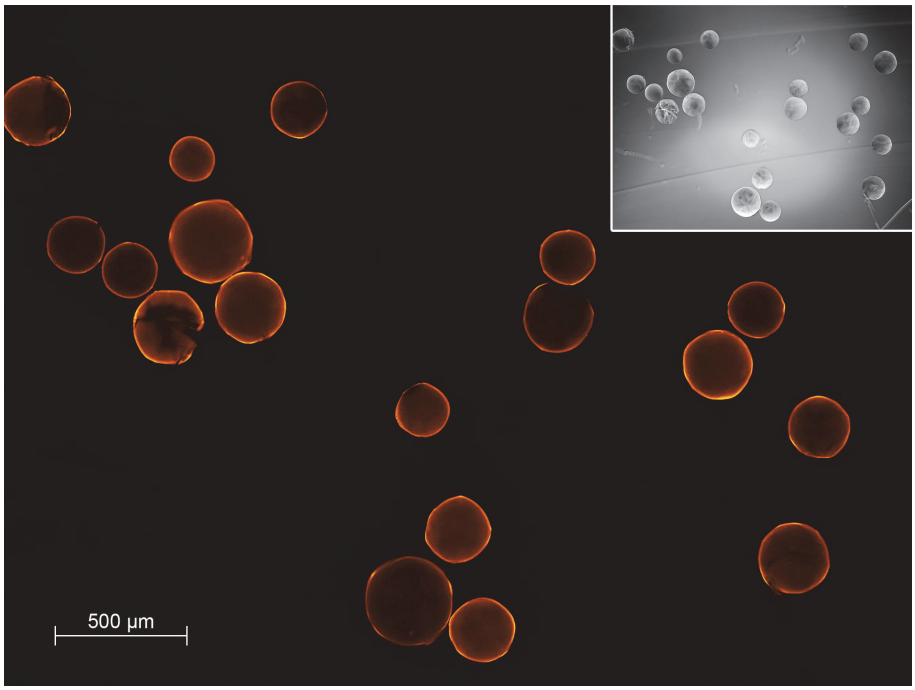


Figure S32. Fluorescence microscopic images of Testpep$_1$ resin after enzymatic activation and ligation with the fluorescent probe (offset transmission mode).

6.5.6 Autofluorescence of Testpep$_2$

1.5 mg of Testpep$_2$ resin (FGG-GGGM) was swollen in Milli-Q water for more than one hour and afterwards examined under the fluorescence microscope. Very low fluorescence intensity was observed.

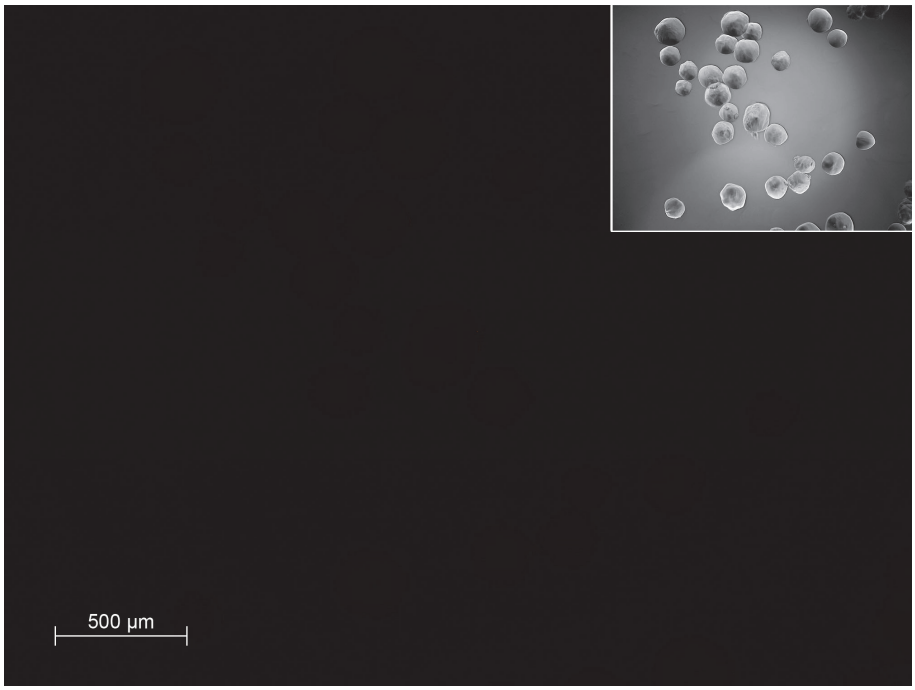


Figure S33. Fluorescence microscopic images of Testpep$_2$ resin (offset transmission mode).

6.5.7 Interaction of Testpep$_2$ with the fluorescent probe

1.0 mg of Testpep$_2$ resin (0.33 µmol. FGG-GGGM) was swollen in Milli-Q water for more than one hour. Afterwards the resin was incubated in a solution of 0.16 mg (0.17 µmol. 0.5 eq) fluorescent probe in 1 mL Milli-Q water for 5 min. Subsequently the resin was washed with MeOH (3x) and Milli-Q water (2x) and examined under the fluorescence microscope. Slightly increased fluorescence intensity could be observed compared to the autofluorescence. Still intensity is low enough that the effects resulting from interaction with the fluorescent probe can be neglected.

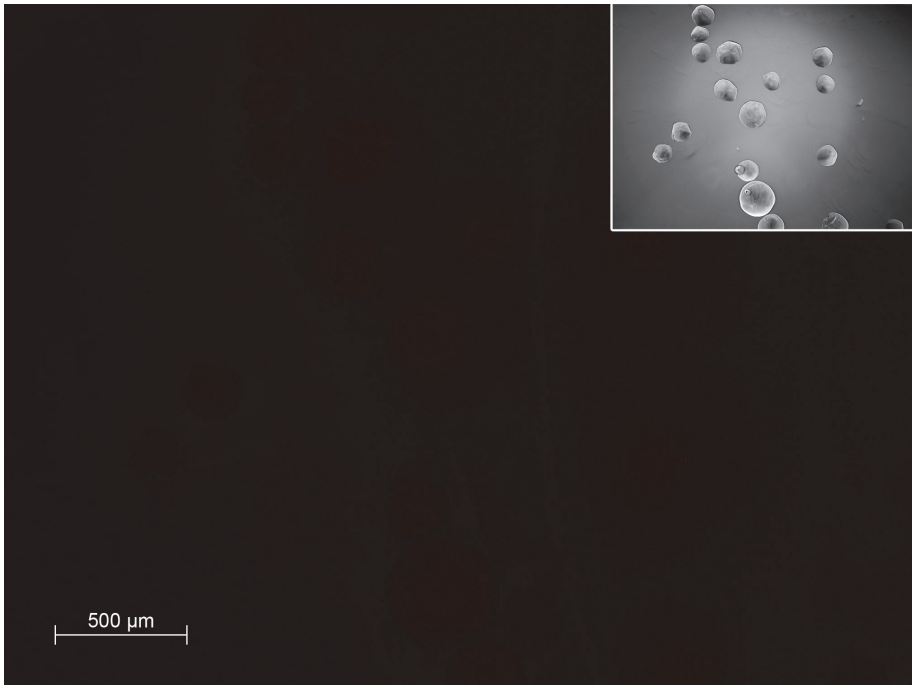


Figure S34. Fluorescence microscopic images of Testpep$_2$ resin after incubation with the fluorescent probe (offset transmission mode).

6.5.8 Activation and labeling of Testpep$_2$

1.0 mg of Testpep$_1$ resin (0.33 µmol. FGG-GGGM) was enzymatically activated and labeled according to protocol 3.7 using 0.16 mg (0.17 µmol. 0.5 eq) fluorescent probe. Low fluorescence intensity is observed suggesting some kind of interaction of the components. By comparison with activation experiment 6.5.5 it is clear that no covalent ligation of the fluorescent probe has taken place and the enzymatic oxidation only affects tyrosine.

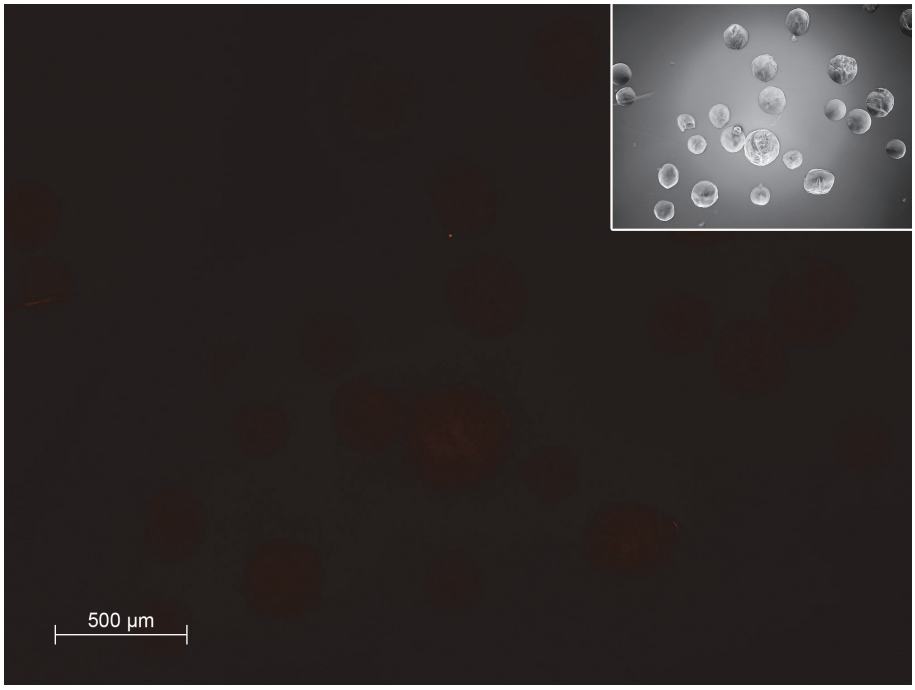


Figure S35. Fluorescence microscopic images of Testpep$_2$ resin after enzymatic activation in the presence of the fluorescent probe (offset transmission mode).

6.5.9 Activation and labeling of Testpep$_1$ and Testpep$_2$

Both Testpeps were simultaneously exposed to tyrosinase as well as the fluorescent probe and were activated according to protocol 3.7. 0.5 mg of each Testpep (total of 0.31 µmol) and 0.15 mg (0.16 µmol. 0.5 eq) of the fluorescent probe were used for activation. Selectivity of the labeling process is shown by the presence of resin beads with high and low fluorescence intensity. Testpep$_1$, containing tyrosine, was oxidized by the enzyme and a ligation with the fluorescent probe took place selectively in the presence of Testpep$_2$, which cannot be oxidized by the enzyme. These findings can be transferred to the more complex peptide library.

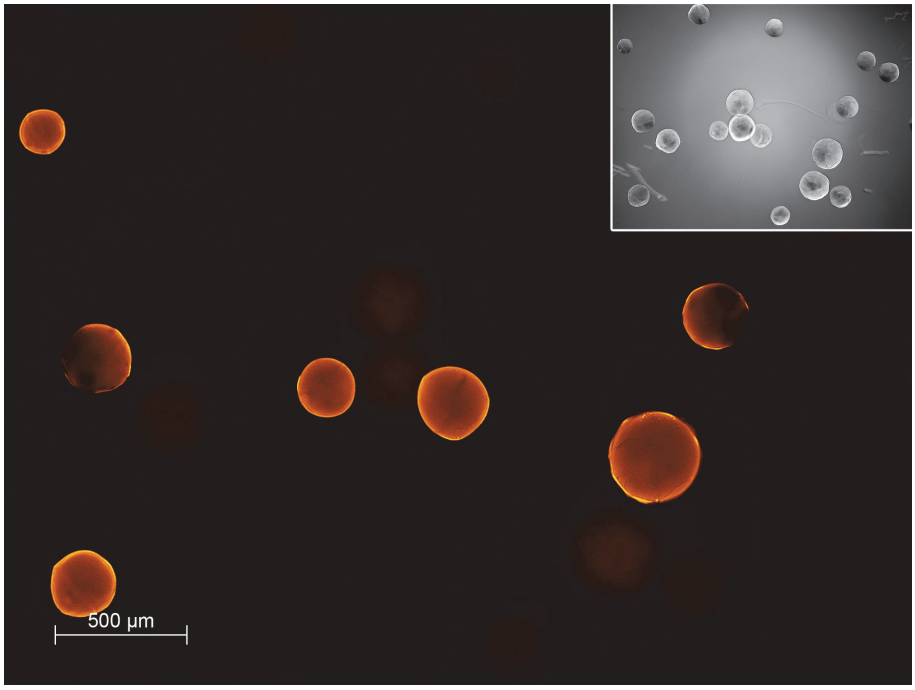


Figure S36. Fluorescence microscopic images of a mixture of Testpep$_1$ and Testpep$_2$ resin after enzymatic activation in the presence of the fluorescent probe (offset transmission mode).

6.5.10 Autofluorescence of the peptide library

0.9 mg of peptide library resin was swollen in Milli-Q water for more than one hour and afterwards examined under the fluorescence microscope. No fluorescence was observed.

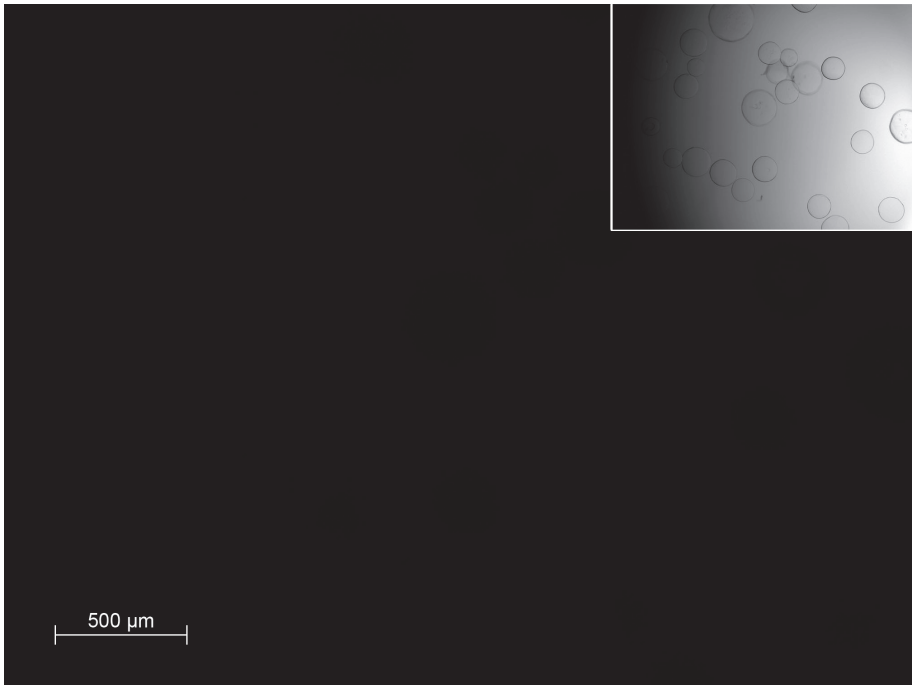


Figure S37. Fluorescence microscopic images of peptide library resin (offset transmission mode).

6.5.11 Interaction of the fluorescent probe with the peptide library

0.6 mg of peptide library resin (0.26 µmol) was swollen in Milli-Q water for more than one hour. Afterwards the resin was incubated in a solution of 0.12 mg (0.13 µmol. 0.5 eq) fluorescent probe in 1 mL Milli-Q water for 5 min. Subsequently the resin was washed with MeOH (3x) and Milli-Q water (2x) and examined under the fluorescence microscope. Very low fluorescence intensity can be observed. It can be deduced that there is no significant interaction between the fluorescent probe and the peptide library beads.

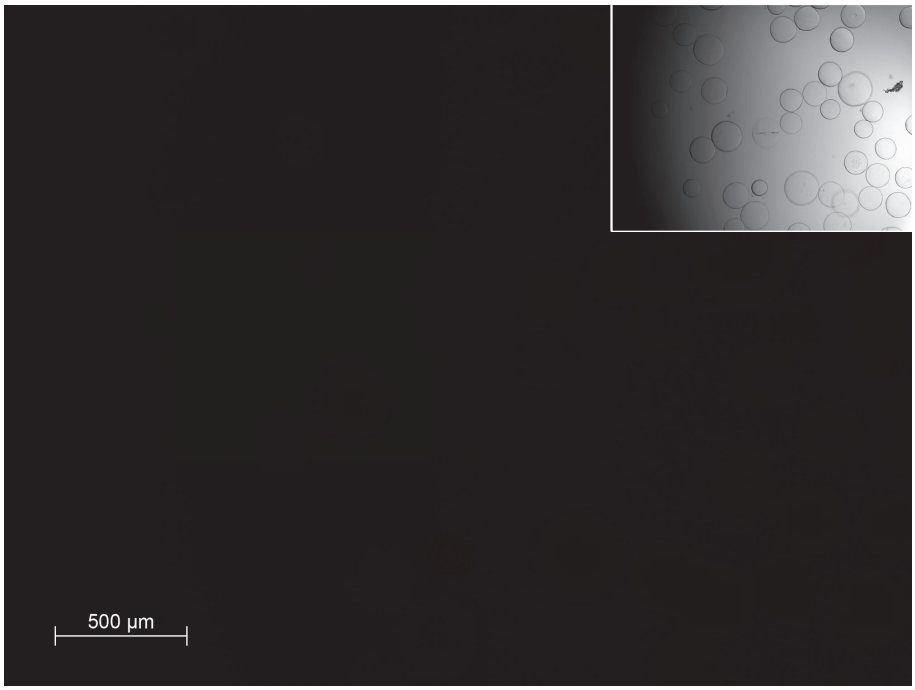


Figure S38. Fluorescence microscopic images of peptide library resin after incubation with the fluorescent probe (offset transmission mode).

6.5.12 Activation and labeling of the peptide library

1.0 mg of the peptide library resin (0.44 µmol) was enzymatically activated and labeled according to protocol 3.7 using 0.21 mg (0.22 µmol. 0.5 eq) fluorescent probe. The image shows one resin bead with high fluorescence intensity. one bead with low intensity and several beads of intermediate intensity reflecting the different reaction rates for enzymatic oxidation of tyrosine.

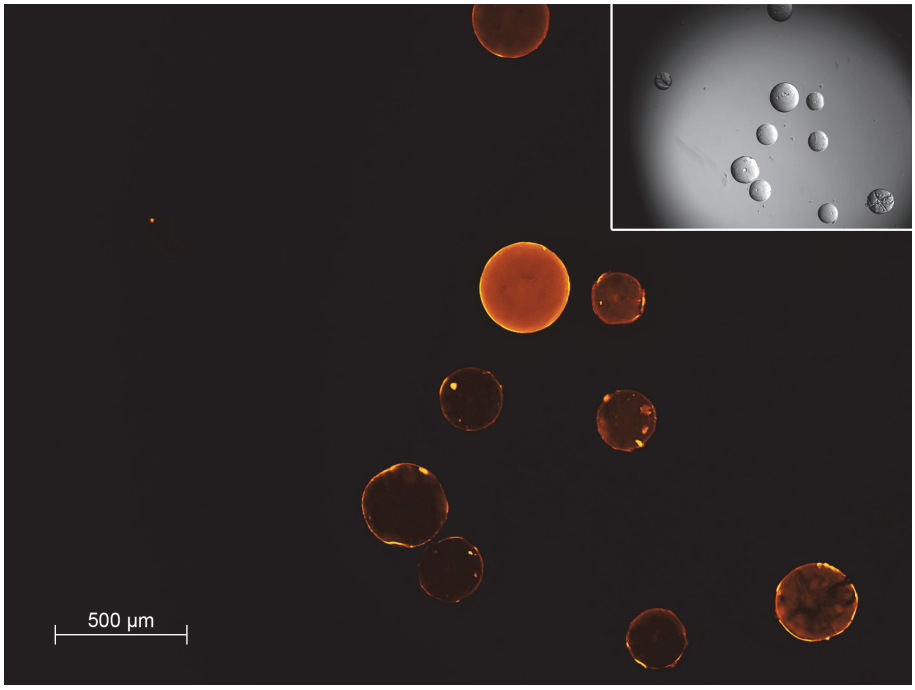


Figure S39. Fluorescence microscopic images of peptide library resin after enzymatic activation in the presence of the fluorescent probe (offset transmission mode).

7. References

1. Duckworth, H. W.; Coleman, J. E., Physicochemical and Kinetic Properties of Mushroom Tyrosinase. *J. Biol. Chem.* **1970,** *245*, 1613-1625.