

Supplementary Information

Exploring the effect of native and artificial peptide modifications on silaffin induced silica precipitation

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1. General methods

All used solvents and reagents were purchased from commercial sources and used without further purification: *N,N*-dimethylformamide (DMF), dichloromethane (DCM) and acetonitrile (ACN) from Biosolve. 2-(1H-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HBTU) and Fmoc-protected amino acids from Novabiochem. Methanol and trifluoroacetic acid (TFA) from Roth. Ammonium molybdate tetrahydrate, dibenzyl-*N,N*-diisopropylphosphoramidite (BzlO)₂PN(*i*-Pr)₂, Diisopropylethylamine (DIEA), 1,1'-carbonyldiimidazole (CDI), fuming HCl, 1-Hydroxybenzotriazole (HOBt), 4-chloro-7-nitro-1,2,3benzoxadiazole (NBD-Cl), 1,8-octandithiol (ODT), phenol, piperidine, spermidine, *t*-butylhydroperoxide (*t*BuOOH, 5.5 M in decane), triisopropylsilane (TIS), tetramethoxysilane and tetrazole (0.45 M in ACN) from Sigma Aldrich. Succinic anhydride from VWR.

Analytical RP-HPLC analysis was performed on a Beckman (System Gold) instrument using a C4 column (150 x 4.6 mm, 5 μm particle size, Grace Vydac) at a flow rate of 1 mL/min with a gradient from 5 % to 65 % buffer B in buffer A over 30 min (buffer A: 0.1 % (v/v) TFA in ddH₂O, buffer B: 0.08 % (v/v) TFA in ACN).

For reversed phase purification of crude peptides a Varian Pro Star system was used. According to the amount and hydrophobicity of the peptides to be purified, different columns were used: C4 column (250 x 22 mm, 5 μm particle size, Protein C4, Grace Vydac), C4 column (250 x 10 mm, 5 μm particle size, Protein C4, Grace Vydac) and C18 column (250 x 10 mm, 5 μm particle size, Kromasil). Buffer A and B were used as eluents.

Ion exchange chromatography was carried out on a Beckman (System Gold) instrument using a Source 15 Q column (100 x 4.6 mm, Pharmacia Biotech). Elution of peptides from the column was achieved running a linear gradient from 0 % to 25 % of buffer D (20 mM Tris/HCl, 1 M NaCl in ddH₂O, pH 7.8) in buffer C (20 mM Tris/HCl in ddH₂O, pH 7.8) in 30 min at a flow rate of 1 mL/min. After lyophilisation of peptide containing fractions, peptides were dissolved in water and desalted following the RP-HPLC protocol described above.

Mass spectra were acquired by electrospray ionization (ESI-MS) in positive ion mode using a LCQ-classic (Finnigan) or a LCQ-fleet (Thermo Fisher). Liquid chromatography-mass spectrometry (LC-MS) was performed on a LCQ-fleet (Thermo Fisher) connected to an UltiMate3000 Dionex HPLC. Separation was achieved using a BioBasic-4 column (150 x 2.1 mm, 5 μm particle size, Thermo Fisher) at a flow rate of 0.35 mL/min. ddH₂O (+ 0.1 % (v/v) formic acid) and ACN (+ 0.1 % (v/v) formic acid) were used as solvents. Peptide masses were detected using electrospray ionization MS in the positive mode.

Detection for all chromatographic methods occurred at 214 and 280 nm wavelength.

2. General protocol for solid phase peptide synthesis

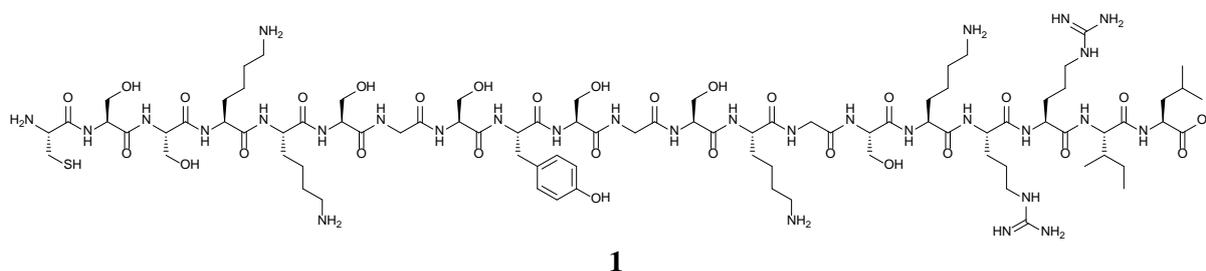
All peptides were synthesised manually on solid support using fluorenylmethoxycarbonyl (Fmoc) chemistry.¹ Syntheses were performed on 0.2 mmol scale using preloaded Fmoc-Leucine-Wang-polystyrene (Fmoc-Leu-Wang-PS) resin (Novabiochem). Deprotection of the N-terminal Fmoc-group was achieved by treating the resin twice with 20 % (v/v) piperidine in DMF for 3 and 7 min, respectively.

For each amino acid coupling 2.5 eq. of the corresponding Fmoc-amino acid were activated with 2.38 eq. HBTU and 5 eq. DIEA for 3 min and added to the resin for 30 min. At times coupling and deprotection reactions were checked by ninhydrin tests.² Between coupling and deprotection steps the peptidyl-resin was washed with DMF. Amino acid side chains were protected by base-stable groups as follows: Cys(tButhio) and Cys(tBu), Lys(Boc) and Lys(Mtt), Arg(Pbf), Ser(tBu) and Ser(Trt), Tyr(tBu). To improve synthesis yields, the pseudo-proline dipeptide Fmoc-Gly-Ser($\psi^{\text{Me,Me}}\text{pro}$)-OH was used as a building block.^{3,4} After completion of peptide elongation the N-terminal Fmoc group was removed, the peptidyl resin was washed with DCM and MeOH and dried under vacuum.

Overall deprotection and cleavage of peptides from resin were achieved with 5 % TIS, 2.5 % ddH₂O and 92.5 % TFA or with a mixture of 5 % phenol, 5 % TIS, 2.5 % ODT, 5 % ddH₂O and 82.5 % TFA (10 mL/g resin) for 3 h at RT. Crude peptides were precipitated by addition of cold diethylether and subsequent centrifugation. Precipitated peptides were washed twice with ether and after removal of the supernatant dissolved in 50 % acetonitrile in water and finally lyophilized. Calculation of yields for purified peptides are based on the synthesis scale.

3. Synthesis notes and analytical data for silaffin peptides

3.1 Peptide 1 H-CSSKKSYSYSGSKGSKRRIL-OH



The unmodified silaffin R5 peptide **1** was synthesised using standard SPPS procedures as described above. Final deprotection and cleavage of the peptide from the resin was accomplished with 5 % TIS, 2.5 % ddH₂O and 92.5 % TFA. The peptide was purified by reversed phase HPLC (preparative C4 column, 10 mL/min flow rate, linear gradient from 5 % to 65 % buffer B in buffer A in 60 min). 27.9 mg of pure peptide were obtained from 100 mg (26.5 μmol) of peptidyl resin (49.8 % yield).

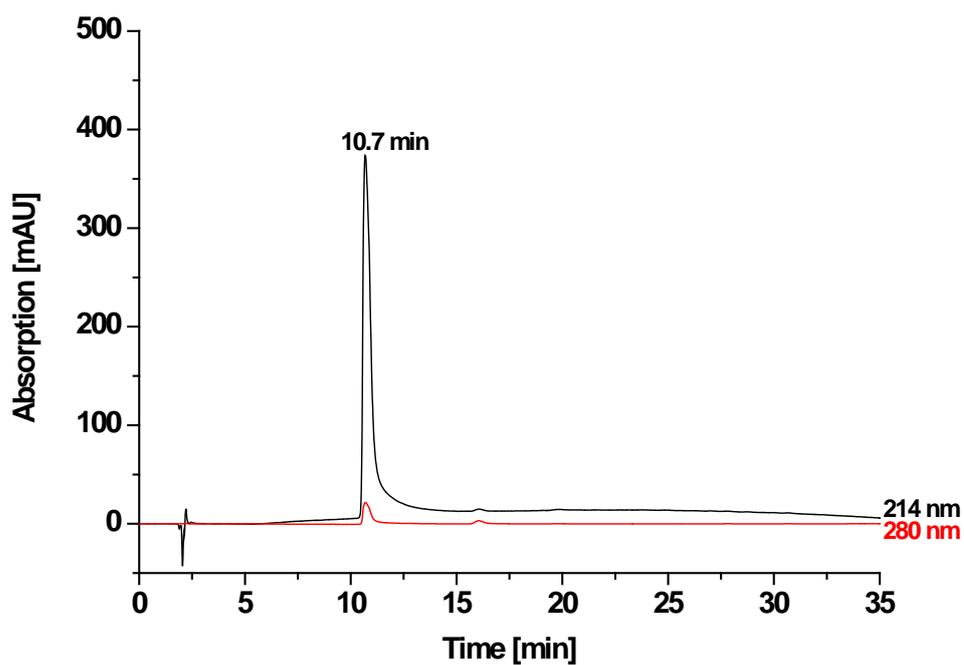


Figure 1: Analytical HPLC chromatogram of purified peptide **1**.

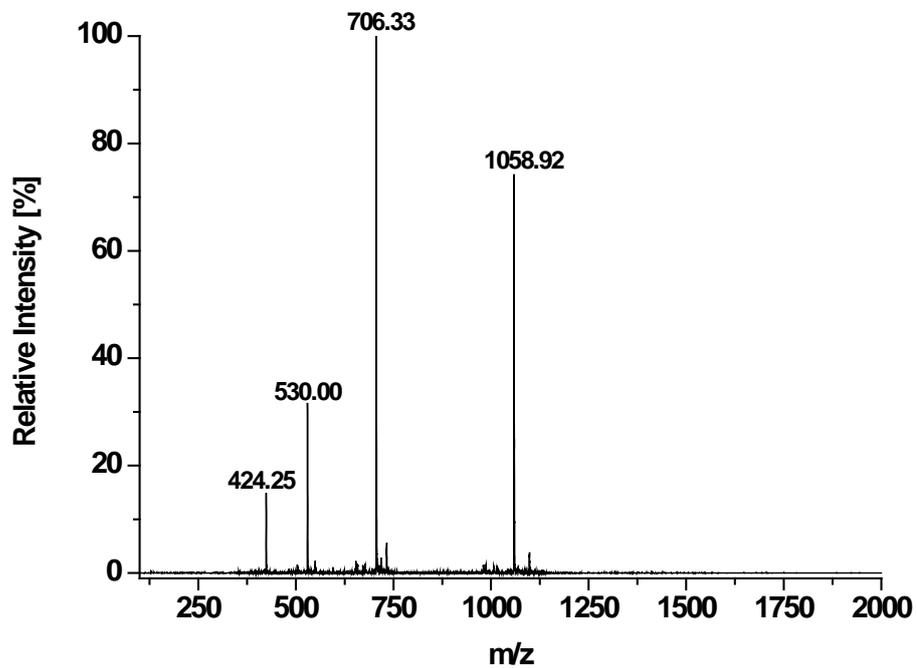
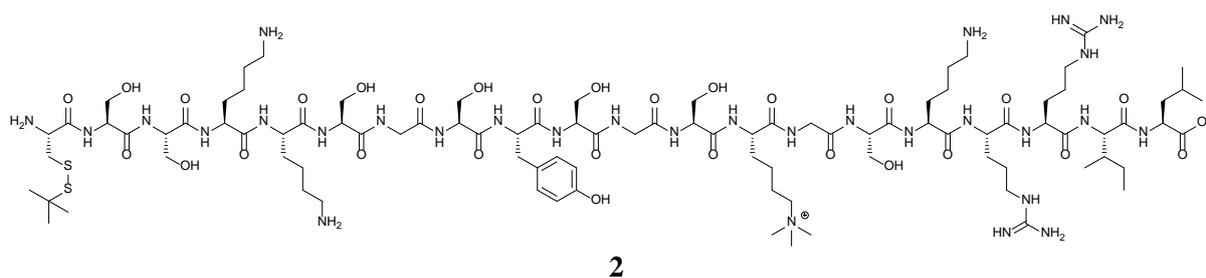


Figure 2: ESI-MS of purified peptide **1**. Mass calculated for $C_{87}H_{155}N_{30}O_{29}S$: 2116.13 $[M+H]^+$, found: 1058.92 $[M+2H]^{2+}$, 706.33 $[M+3H]^{3+}$, 530.00 $[M+4H]^{4+}$, 424.25 $[M+5H]^{5+}$.

3.2 Peptide 2 H-CSSKKSYSYSGSK(Me)₃GSKRRIL-OH



Peptide **2** was synthesised following the general protocol using N- α -Fmoc-N- ϵ -trimethyl-N-L-lysine (Novabiochem) as building block at position 13. Peptide deprotection and cleavage from the solid support was done with 5 % TIS, 2.5 % ddH₂O and 92.5 % TFA. The crude peptide was purified via reversed phase chromatography (preparative C4 column, 10 mL/min flow rate, linear gradient from 20 % to 50 % buffer B in buffer A in 20 min) yielding 20.7 mg of peptide **2** in good purity (18.9 % yield from 194 mg (48.8 μ mol) peptidyl resin).

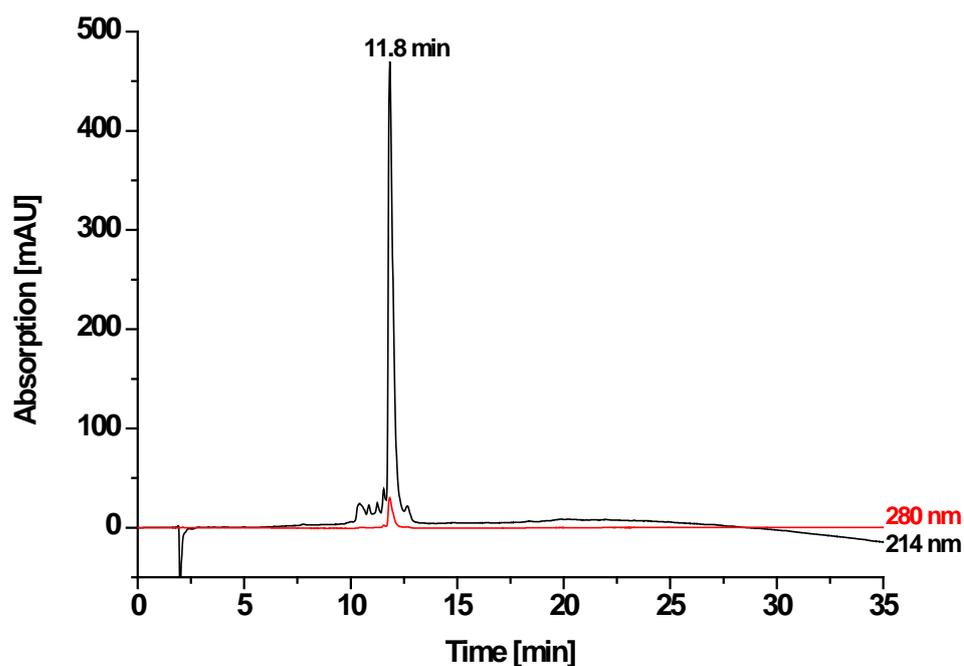


Figure 3: Analytical HPLC chromatogram of purified peptide **2**.

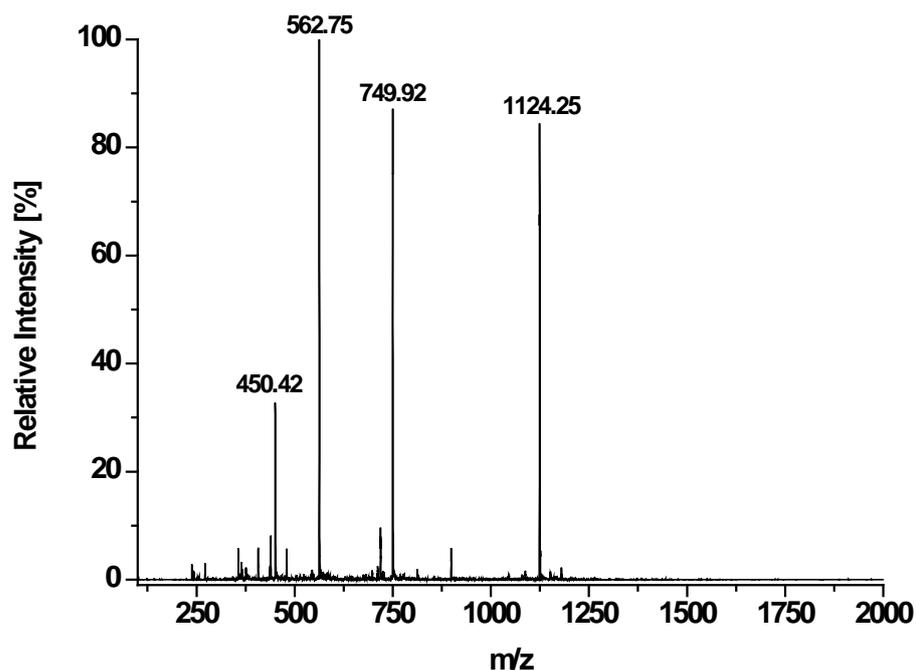
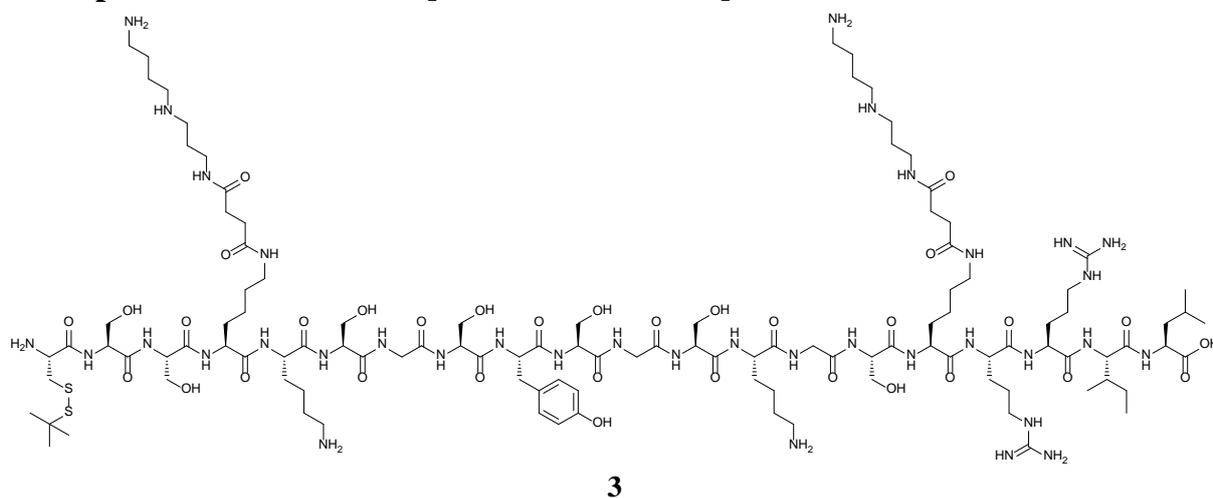


Figure 4: ESI-MS of purified peptide **2**. Mass calculated for $C_{94}H_{170}N_{30}O_{29}S_2^{+}$: 2247.22 $[M+H]^+$, found: 1124.25 $[M+2H]^{2+}$, 749.92 $[M+3H]^{3+}$, 562.75 $[M+4H]^{4+}$, 450.42 $[M+5H]^{5+}$.

3.3 Peptide 3 H-CSSK(Sp)KSGSYSGSKGSK(Sp)RRIL-OH



Modification of the silaffin R5 peptide with the polyamine spermidine was achieved by synthesizing the native sequence using Fmoc-Lys(Mtt)-OH at positions 4 and 16. Mtt-protecting groups were selectively removed by repeated treatment of the peptidyl resin with a solution of 1 % TFA and 1 % TIS in DCM for 2 min and subsequent washing with DCM for 1 min. 219 mg (0.05 mmol) peptidyl resin were swollen again in DMF for 1 h. A solution of 100 mg (0.5 mmol, 10 eq.) succinic anhydride and 150 μ L DIEA in 1.5 mL 0.5 M HOBt in DMF was added to the resin for 30 min. After 30 min activation of the carboxyl group with CDI (10 mL, 0.5 M in DMF), spermidine (726 mg, 5.0 mmol, 100 eq.) dissolved in a solution of HOBt (5mL, 0.5 M in DMF) was added to the resin and incubated

at room temperature for 30 min. The peptidyl resin was washed and dried under vacuum. Peptide **3** was deprotected and cleaved from the resin using a mixture of 5 % phenol, 5 % TIS, 2.5 % ODT, 5 % ddH₂O and 82.5 % TFA. Pure peptide **3** was obtained after RP-HPLC purification (semipreparative C4 column, 3 mL/min flow rate, linear gradient from 5 % to 65 % buffer B in buffer A in 60 min) with a yield of 4.1 mg pure peptide from 211 mg (0.05 mmol) of peptidyl resin (3.1 % yield).

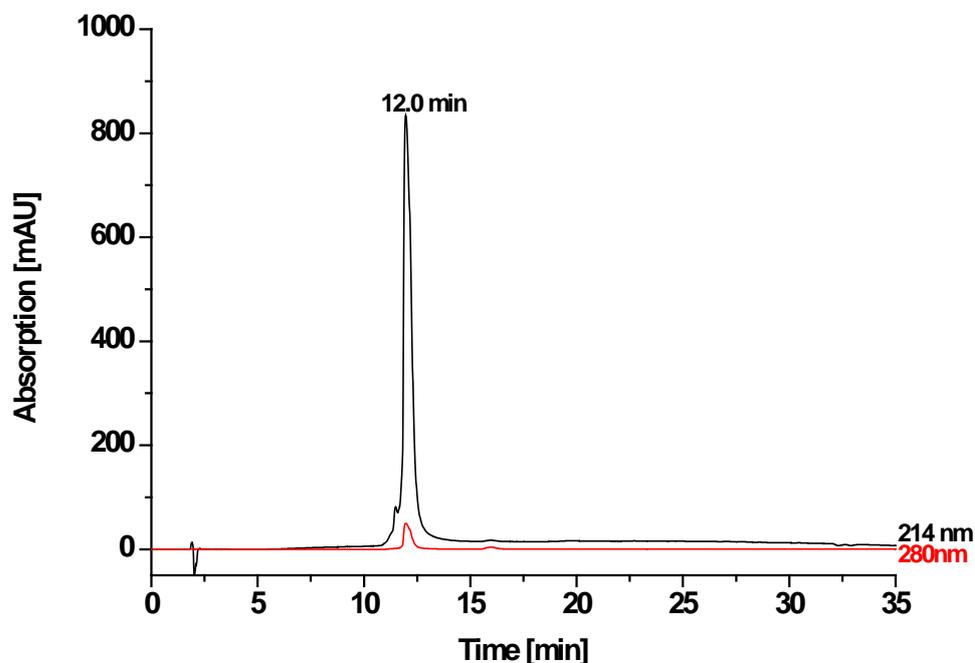


Figure 5: Analytical HPLC chromatogram of purified peptide **3**.

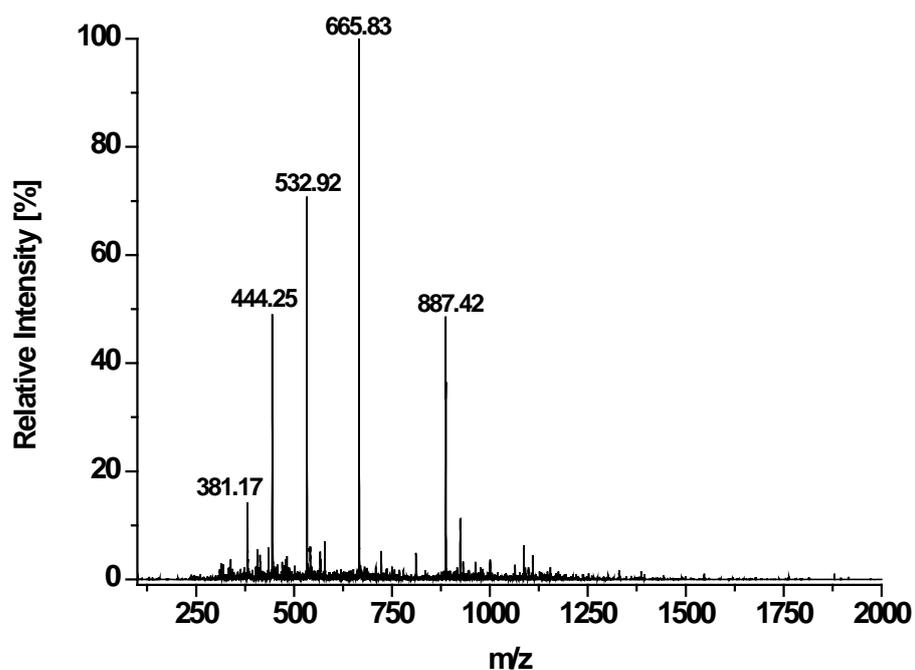
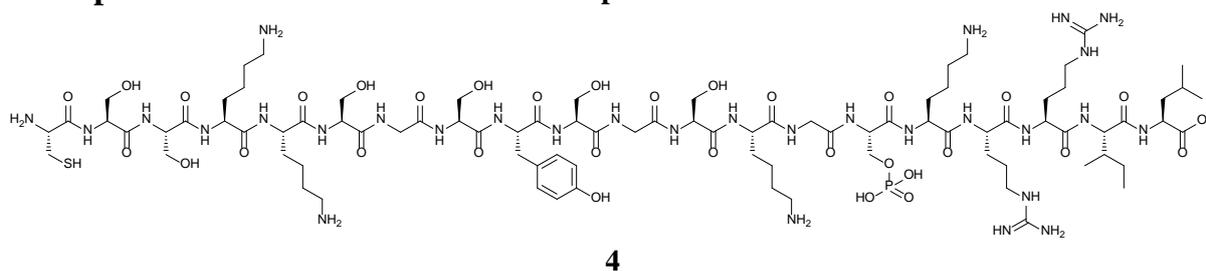


Figure 6: ESI-MS of purified peptide **3**. Mass calculated for C₁₁₃H₂₀₅N₃₆O₃₃S₂⁺: 2658.49 [M+H]⁺, found: 887.42 [M+3H]³⁺, 665.83 [M+4H]⁴⁺, 532.92 [M+5H]⁵⁺, 444.25 [M+6H]⁶⁺, 381.17 [M+7H]⁷⁺.

3.4 Peptide 4 H-CSSKKS^GSYSGSK^{Gp}SKRRIL-OH



Silaffin peptide **4**, carrying a single phosphorylated serine residue, was assembled following the general protocol. At position 15 the commercial available phosphoserine building block *N*- α -Fmoc-O-benzyl-L-phosphoserine (Novabiochem) was incorporated. Peptide **4** was cleaved from the resin with 5 % TIS, 2.5 % ddH₂O and 92.5 % TFA and purified by reversed phase HPLC (preparative C4 column, 10 mL/min flow rate, 20 % to 50 % buffer B in buffer A in 20 min). From 100 mg (24.9 μ mol) peptidyl-resin 21.2 mg pure peptide **4** were isolated (38.8 % yield).

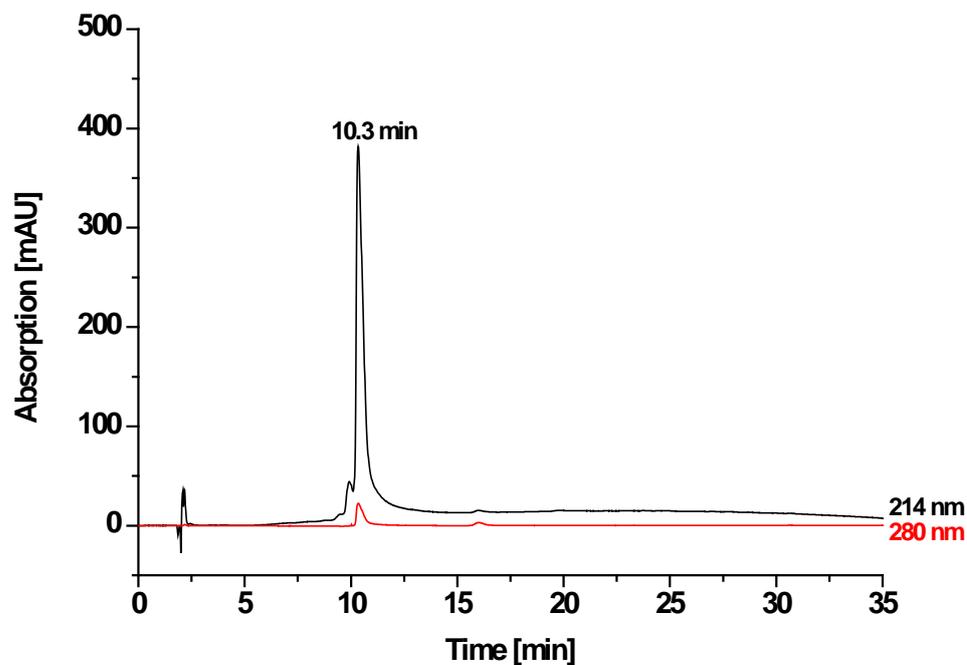


Figure 7: Analytical HPLC chromatogram of purified peptide **4**.

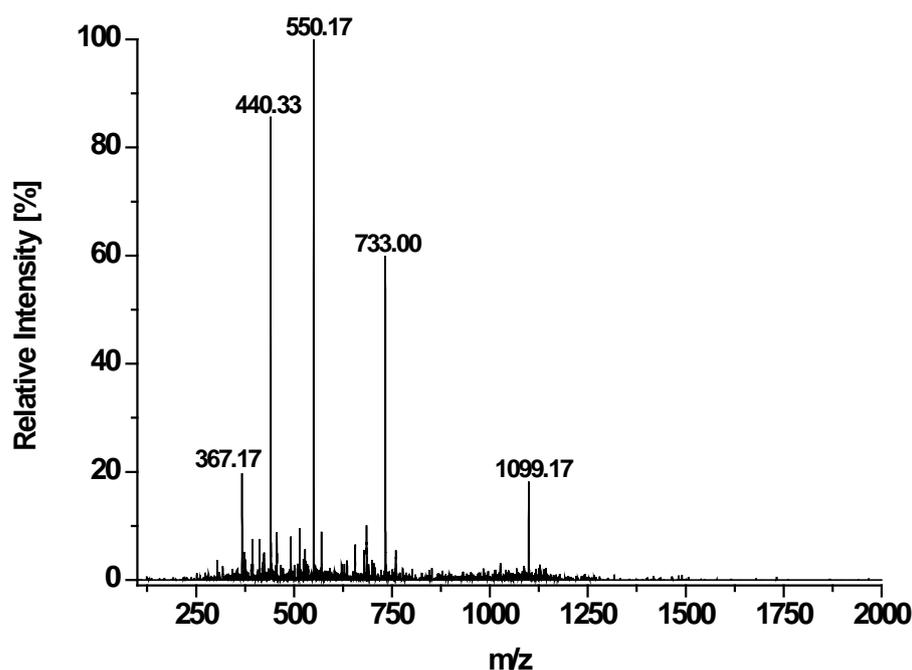
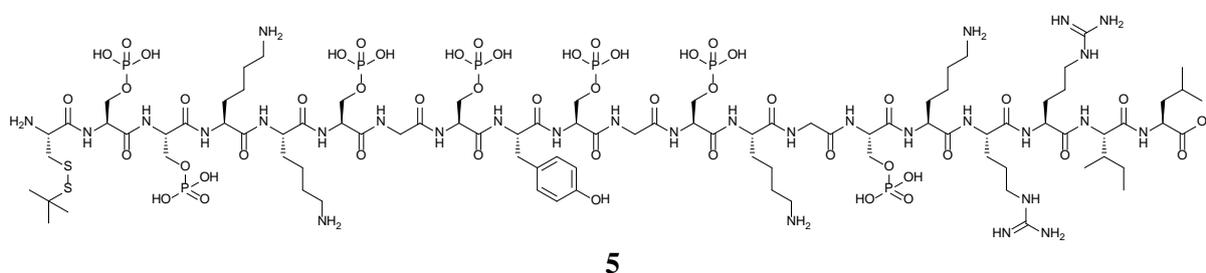


Figure 8: ESI-MS of purified peptide **4**. Mass calculated for $C_{87}H_{156}N_{30}O_{32}PS$: 2196.10 $[M+H]^+$, found: 1099.17 $[M+2H]^{2+}$, 733.00 $[M+3H]^{3+}$, 550.17 $[M+4H]^{4+}$, 440.33 $[M+5H]^{5+}$, 367.17 $[M+6H]^{6+}$.

3.5 Peptide 5 H-CpSpSKKpSGpSYpSGpSKGpSKRRIL-OH



5

Peptide **5** was obtained by global phosphorylation^{5, 6, 7} of all seven unprotected serine-hydroxyl groups occurring in the silaffin sequence. The peptide backbone was assembled on the solid support following the general protocol and by incorporating trityl-protected serine residues (Fmoc-Ser(Trt)-OH). The *N*-terminal cysteine residue was Boc-protected (Boc-Cys(StBu)-OH) in order to avoid β -elimination during basic Fmoc-deprotection after post-synthetic phosphorylation on the solid support. After completion of chain elongation the Trt-protecting groups were selectively removed by repeated treatment of peptidyl-resin (243 mg, 0.05 mmol) with a solution of 1 % TFA and 1 % TIS in DCM for 2 min and subsequent washing with DCM for 1 min. The peptidyl resin was placed in a suitable reaction vessel and dried under vacuum overnight. The following reactions were carried out under an argon atmosphere and with dried solvents. The peptidyl-resin was swollen in 3.6 mL dry DMF for 2 h. After addition of tetrazole (5.7 mL, 0.45 M in ACN, 2.6 mmol) and $(BzlO)_2PN(i-Pr)_2$ (0.39 mL, 1.04 mmol) the reaction vessel was gently agitated for 3 h at RT and then washed with dry DMF (5 x 10 mL). For oxidation of the intermediate phosphite ester 1.9 mL dry DMF and *t*-butyl hydroperoxide (2.4 mL, 5.5 M in decane, 13.2 mmol) were added for 1.5 h. Finally, the resin was washed with DMF

and dried under vacuum. Final deprotection and cleavage of the peptide from the resin was accomplished with 5 % TIS, 2.5 % ddH₂O and 92.5 % TFA. Crude peptide was first purified by reversed phase HPLC (semipreparative C18 column, 3 mL/min flow rate, 20 % to 50 % buffer B in buffer A in 20 min) followed by ion exchange chromatography resulting in highly pure peptide **5** (0.8 mg from 0.05 mmol peptidyl resin, 0.6 % yield).

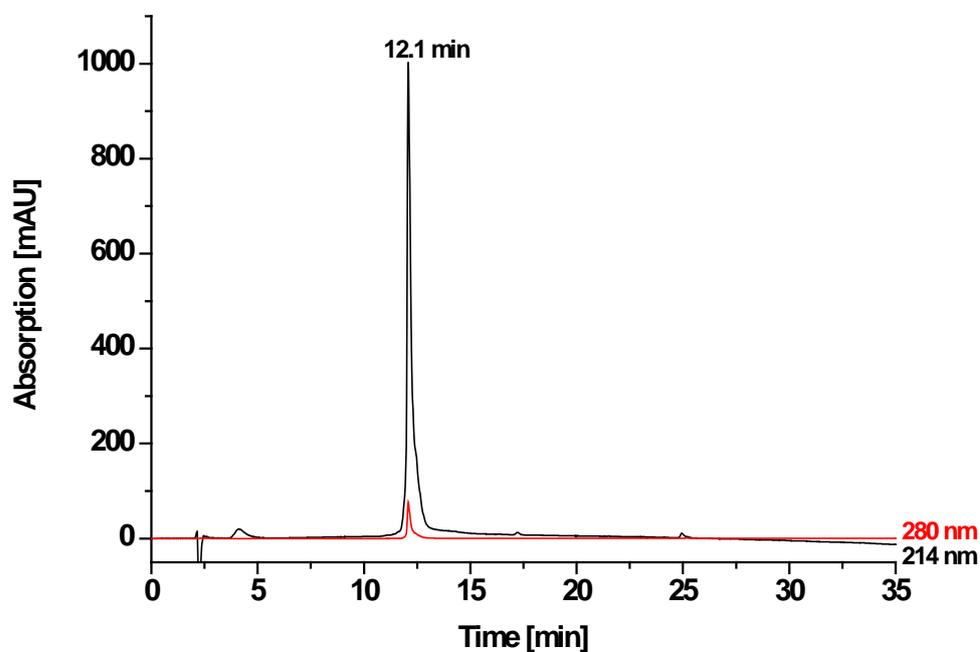


Figure 9: Analytical HPLC chromatogram of purified peptide **5**.

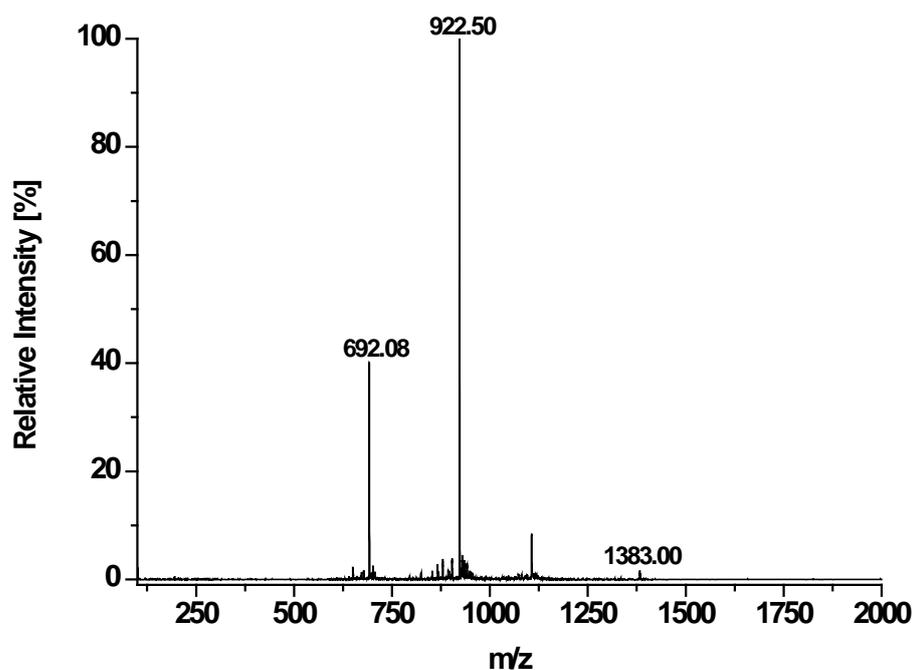
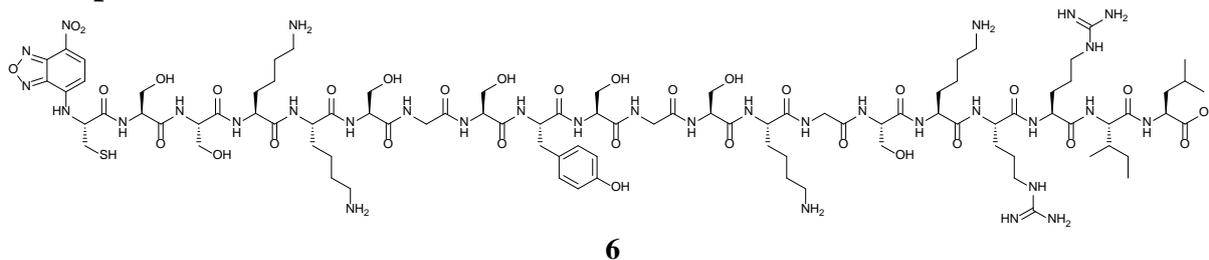


Figure 10: ESI-MS of purified peptide **5**. Mass calculated for C₉₁H₁₇₀N₃₀O₅₀P₇S₂: 2763.93 [M+H]⁺, found: 1383.00 [M+2H]²⁺, 922.50 [M+3H]³⁺, 692.08 [M+4H]⁴⁺.

3.6 Peptide 6 NBD-CSSKKSGSYSGSKGSKRRIL-OH



A part of the unmodified peptidyl-resin (see synthesis of peptide **1**) was used for N-terminal fluorescent labelling with nitrobenzoxadiazole (NBD). Peptidyl-resin **1** (150 mg, 0.04 mmol) was swollen in DMF for 2 h. 4-chloro-7-nitrobenzofurazan (77.8 mg, 0.4 mmol, 10 eq.) and DIEA (7.0 μ L, 0.04 mmol, 1 eq.) were dissolved in 400 μ L DMF. The obtained solution was added to the peptidyl-resin and incubated at 37 $^{\circ}$ C for 16 h under permanent shaking. After extensive washing of the resin with DMF, DCM and MeOH the peptidyl-resin was dried under vacuum. Finally the fluorescently labelled peptide was cleaved from the resin with 5 % TIS, 2.5 % ddH₂O and 92.5 % TFA. Peptide **6** was purified by reversed phase HPLC (preparative C4, 10 mL/min flow rate, 5 % to 65 % buffer B in buffer A in 60 min, and subsequent semipreparative C18 column, 3 mL/min flow rate, 5 % to 65 % buffer B in buffer A in 60 min) yielding 3.8 mg of peptide **6** in satisfactory purity (4.2 % yield from 0.04 mmol peptidyl resin).

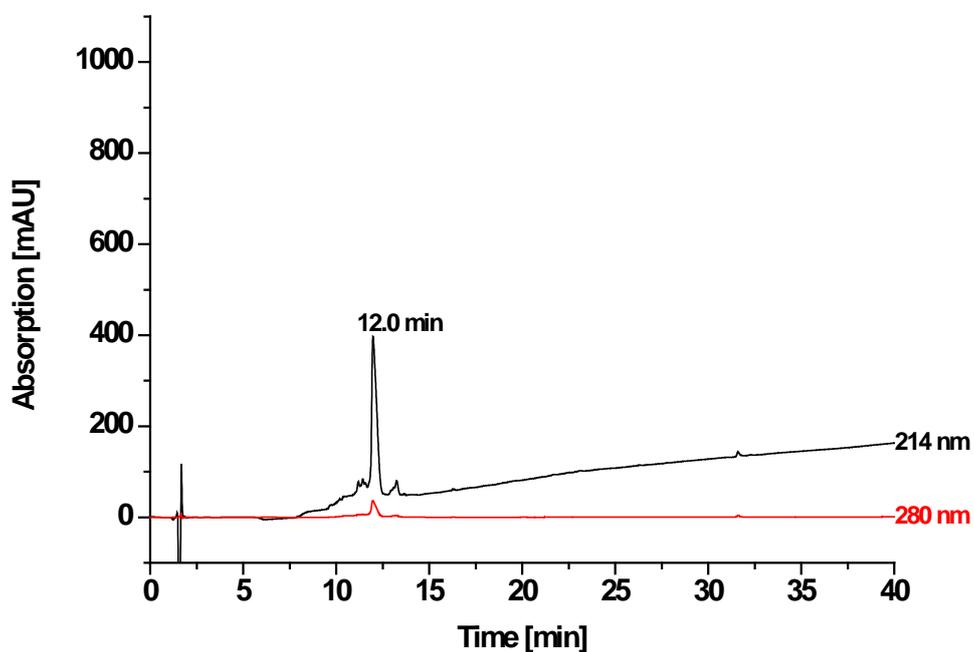


Figure 11: Analytical HPLC chromatogram of purified peptide **6**.

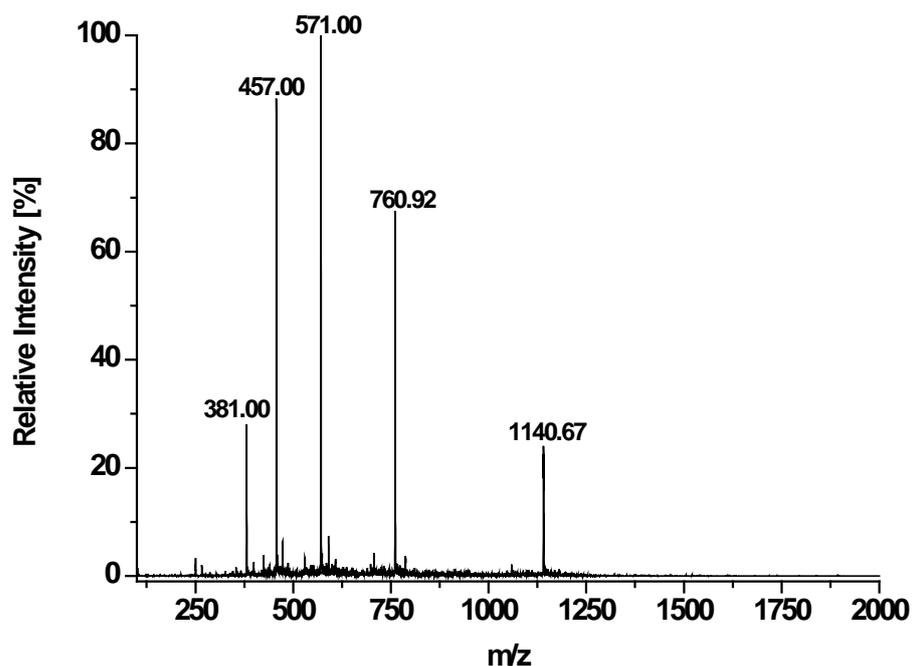


Figure 12: ESI-MS of purified peptide **6**. Mass calculated for $C_{93}H_{156}N_{33}O_{32}S$: 2279.13 $[M+H]^+$, found: 1140.67 $[M+2H]^{2+}$, 760.92 $[M+3H]^{3+}$, 571.00 $[M+4H]^{4+}$, 457.00 $[M+5H]^{5+}$, 381.00 $[M+6H]^{6+}$.

4. *In vitro* silica precipitation assays

4.1 *In vitro* silica precipitation and microscopic analysis

Peptides to be analysed were dissolved to a final concentration of 1 mg/mL in 50 mM potassium phosphate buffer at pH 7.0. Silicic acid was generated by hydrolysis of 250 mM tetramethoxysilane in 1 mM aqueous HCl for 4 min. Silica precipitation reactions were initiated by addition of silicic acid to peptide solutions to a final concentration of 25 mM. Reactions were incubated at RT for 30 min. Silica precipitates were collected by centrifugation (5 min, $16.873 \times g$) and washed twice with water. Silicic acid solutions without silaffin peptides did not lead to the formation of any precipitate. Silica precipitate collected by centrifugation was suspended in water, applied to a *Thermanox*TM coverslip (Thermo scientific) and air dried. The coverslips were placed onto sample holders and sputter coated with gold in high vacuum (Bal-Tec SCD 005). Electron micrographs were recorded with a scanning electron microscope (JEOL JSM 5900 LV) operating at 20 kV. Analysis of elemental composition was done by energy dispersive X-ray (EDX) spectroscopy (Röntec). For fluorescence microscopy, silica material was suspended in water, applied to a glass slide and covered with a cover glass. Fluorescence micrographs were obtained using a Zeiss Axiovert 200 microscope using the oil-immersion objective.

4.2 Determination of silicon concentration

In vitro silica precipitation assays were performed as described in 4.1. The collected silica precipitates were dissolved in 2 M NaOH for 1 h at RT and quantified by a modified β -silicomolybdate method⁸ as described by Wieneke et al.⁹ Calibration curves are based on a silicon atomic absorption standard solution (Sigma Aldrich). All assays were at least performed in triplicate.

5. Particle size analysis

The statistical analysis of silica particle size distribution was done with ImageJ. For a representative number of silica particles resulting from peptides **1**, **2** and **3** the diameter was determined manually from the corresponding digital electron micrographs. Fig. 13 shows the calculated frequency of particle diameters resulting from peptides **1**, **2** and **3**.

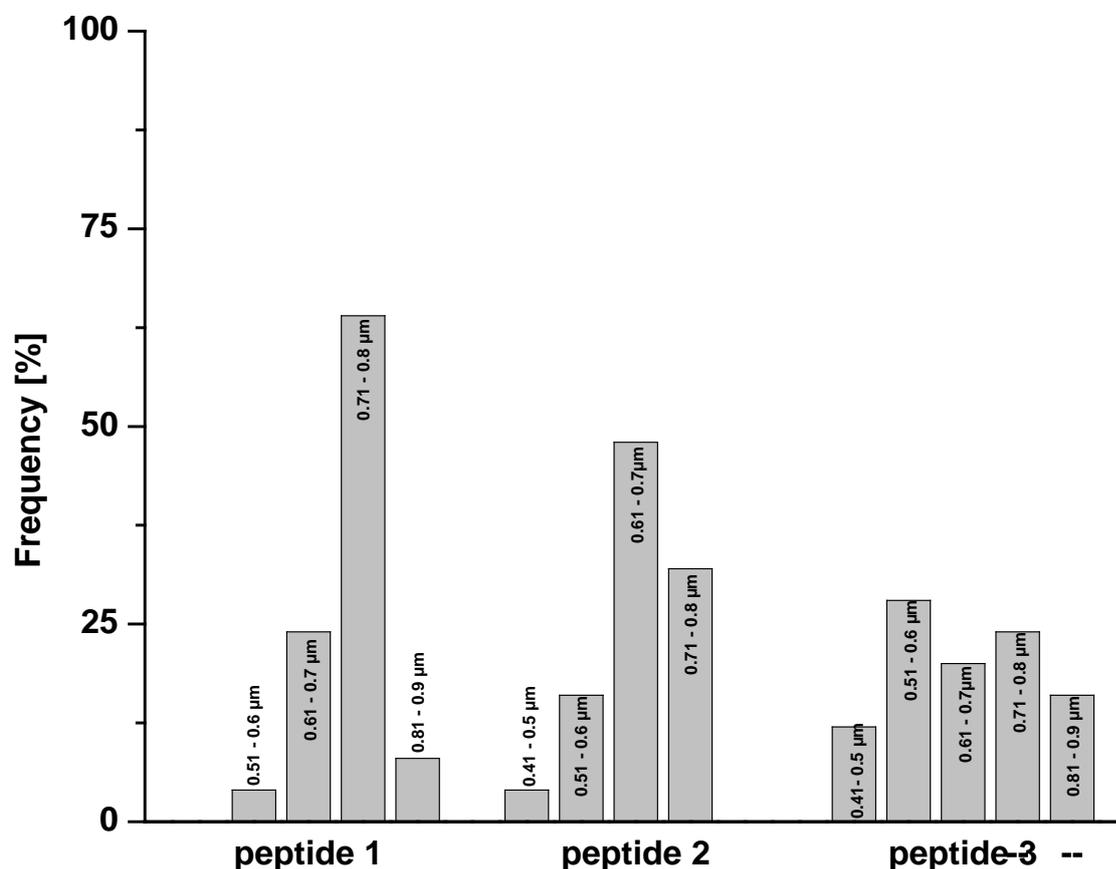


Figure 13: Distribution of silica particle sizes resulting from peptide **1**, **2** and **3** shown as the frequency of particle diameters.

6. References

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