Transmembrane Domain Peptide/Peptide Nucleic Acid Hybrid as a Model of a SNARE Protein in Vesicle Fusion**

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1 Materials and General Methods

1.1 Solvents
All technical solvents were distilled prior to use. The solvents of analytical and HPLC grade were used as supplied. Anhydrous DMF, diethyl ether, THF and DCM were obtained from Aldrich, Fluka or Acros. N-Methyl-2-pyrrolidon (NMP) for peptide synthesis was obtained from Carl Roth GmbH, controlled by GS and used without additional purification. Piperidine was obtained from Riedel de Haen and used as supplied. Acetonitrile and methanol were of HPLC grade.

1.2 Reagents
All chemicals were of the highest grade available and used as supplied. Fmoc-protected amino acids and PNA were purchased from NovaBiochem, GL Biochem (Shanghai) Ltd., ACM Research Chemicals or PANAGENE Inc. (Daejeon) and used with the following side-chain protection: t-Bu (Asp, Glu, Ser, Thr, Tyr), Boc (Lys, Trp), Trt (Asn, Cys, His), Pbf (Arg), Bhoc (A, C, G, T). Resins for SPPS were purchased from Novabiochem (Merck).

1.3 Lyophilization
A Christ Alpha-2-4-lyophilizer equipped with a high vacuum pump was used to liberate dry substances from water.

1.4 High Performance Liquid Chromatography (HPLC)
HPLC was performed on a Pharmacia Äkta basic system (pump type P-900, variable wavelength, detector, type UV-900) using the following columns:

Analytical HPLC:  
Phenomenex: RP C-4 (150 × 4.6 mm, 5 µm)  
Phenomenex: RP C-18 (250 × 4.6 mm, 5 µm)

Semi-Preparative HPLC:  
Phenomenex: RP C-4 (250 × 10 mm, 5 µm)  
Phenomenex: RP C-18 (250 × 10 mm, 5 µm)

All HPLC runs were performed by using a linear gradient of A (0.1 % aq. TFA), B1 (100 % acetonitrile, 0.1 % TFA) and B2 (80 % aq. acetonitrile, 0.1 % aq. TFA) within 30 min. For analysis and purification HPLC grade, acetonitrile and ultra pure water were used. Flow rates were taken as 1 mL/min for the analytical, 3 mL/min for the semi-preparative purpose. All crude samples were dissolved in a acetonitrile-water or acetonitrile-formic acid mixture and
filtered prior to injection. UV detection was conducted at 215 nm for fully deprotected peptides and at 260 nm for PNAs containing peptides.

2 Solid Phase Peptide Synthesis (SPPS)

2.1 Handling of Solid Support and Building Blocks
Amino acid building blocks were stored at -18 °C. For the storage of resins for SPPS the temperature did not exceed 4 °C. Before opening, chemicals were warmed to room temperature. If necessary, both resins and amino acids were additionally dried in high vacuum.

2.2 Loading of the First Amino Acid
Loading of Wang resins (peptide acid) was performed in an acid/base-resistant syringe, equipped with a polyethylene frit. The resin was pre-swollen in DMF for 1 h and was thoroughly washed with the same solvent. To a solution of amino acid derivative (10 equiv) in DCM/DMF (1:1), DIC (5 equiv) was added and the mixture was stirred for 10 min. This mixture and DMAP (0.1 equiv) were subsequently added to the resin and gentle shaking of the reaction vessel was continued for an additional hour. Then, the resin was subsequently washed with DMF, DCM, MeOH and dried \textit{in vacuo}.

If not purchased as the pre-loaded variants, the resins were self-loaded. After resin-loading the loading density was estimated via UV-analysis of the Fmoc-dibenzofulven deprotection product.\textsuperscript{1} If necessary, the loading procedure was repeated until sufficient loading was achieved. When the required loading density was reached, residual free hydroxyl functions were “capped” by treatment with Ac\textsubscript{2}O (2 M), DIEA (0.6 M), HOBt (60 mM) in NMP twice within 5 min.

2.3 Automated Microwave Assisted SPPS
The \textit{Liberty} peptide synthesizer (CEM, Kamp-Lintfort, Germany) equipped with a \textit{Discover} microwave (MW) reaction cavity (CEM) was used for automated peptide synthesis. Microwave application is triggered by the reaction temperature within the reaction vessel. Standard reagents, protocols and procedures were used for deprotection (piperidine/NMP or piperidine/HOBt/NMP; 210 sec, 75 °C, 20 W), coupling (HBTU/HOBt/DIEA/NMP; 300 sec, 75 °C, 20 W) and capping (Ac\textsubscript{2}O/DIEA/HOBt/NMP; 180 sec, 50 °C, 20 W).
The following amino acid building blocks were used in the automated syntheses: Fmoc-Ala-OH, Fmoc-Arg(Pbf)-OH, Fmoc-Asn(Trt)-OH, Fmoc-Cys(Trt)-OH, Fmoc-Gln(Trt)-OH, Fmoc-Gly-OH, Fmoc-Ile-OH, Fmoc-Leu-OH, Fmoc-Lys(Boc)-OH, Fmoc-Met-OH, Fmoc-Phe-OH, Fmoc-Ser(tBu)-OH, Fmoc-Thr(tBu)-OH, Fmoc-Trp(Boc)-OH, Fmoc-Tyr(tBu)-OH, Fmoc-Val-OH.

Double couplings were usually performed for Ala, Arg, Cys, Gly, Ile, Leu, Trp and Val residues. Special care has to be taken for incorporation of Cys residues. In this case, the temperature for MW-application was reduced to 50 °C and milder reagents for deprotection steps (piperidine/HOBt/NMP) were used.

2.4 Manual SPPS of PNA/Peptide Conjugate

The SPPS of peptide/PNA conjugates was carried out manually at 5 µM scale using Wang resin. Resins were swollen in NMP for 20 min. The coupling cycle started with Fmoc-deprotection applying 20 % piperidine in NMP twice within 10 min, followed by washing with NMP (5 times), DCM (5 times) and again NMP (5 times). For each coupling a PNA or amino acid building block excess of four equivalents was used; the solid building blocks were dissolved in stock solutions of the coupling reagents within 5 min before the bases were added. Activators for coupling were HATU/HOAt (3.9 equiv/4.0 equiv) in NMP. DIEA and 2,6-lutidine were used as activator bases. A 0.25 M concentration was used as the final reaction mixture for the building blocks and HOAt. The HATU concentration was adjusted to 0.24 M. The concentration of bases was 0.2 M for DIPEA and 0.3 M for 2,6-lutidine.

Standard coupling time was 60 min for PNA-monomers. Double couplings were performed for the Fmoc-PNA-g(Bhoc)-OH and Fmoc-PNA-c(Bhoc)-OH building blocks. When double coupling was applied, the coupling time was reduced to 45 min. Standard amino acid building blocks were always introduced by double coupling. After washing with 5 % DIEA in NMP (5 times), DCM (5 times), NMP (5 times) capping was fulfilled with Ac₂O/2,6-lutidine/NMP (1/2/7, v/v/v, twice within 5 min). A final wash with 5 % DIEA/NMP (5 times), MTBE (5 times) and NMP (5 times) completed the coupling cycle.

2.5 Cleavage

After synthesis the resins were filtered off and washed sequentially with NMP (5 times), DCM (5 times), MTBE (5 times) and finally with MeOH (5 times). The resins were dried in vacuo und then peptides (PNA/peptide conjugates) were cleaved from the solid support.
The side chain protection groups were removed at the same time with the peptide cleavage, the standard TFA cleavage was applied using the mixture TFA/H$_2$O/TIS (95/2.5/2.5, v/v/v). If sequences contained sensitive Trp or Cys residues the mixture TFA/thioanisole/H$_2$O/EDT/TIS (82.5/5.0/5.0/5.0/2.5, v/v/v/v/v) was applied providing highly effective scavenging. Cleavage reactions were carried out for 2 h at ambient temperature. Cleavage of PNA/peptide conjugates and PNA was normally performed by applying the mixtures of TFA/TIS/EDT/m$_6$cresol (87.5/2.5/5.0, v/v/v), or TFA/m$_6$cresol/TIS (92.5/5.0/2.5, v/v/v), respectively.\cite{3}

2.6 Post-Cleavage Work-Up

After cleavage, the resulting solution was concentrated under reduced pressure. The crude product was then precipitated via treatment with cold MTBE. The resulting suspensions were centrifuged at -5 °C. The supernatant was discarded and the peptide pellet was washed with MTBE several times and dried in vacuo prior to HPLC. For HPLC analyses PNA/peptide samples were dissolved in MeCN/formic acid (5/1, v/v), PNAs were dissolved in H$_2$O.

3 Preparation of Peptide/Lipid Complexes

3.1 Multilamellar Vesicles (MLVs)

Lipids and cholesterol were dissolved in CHCl$_3$ (about 20 mg⋅mL$^{-1}$), mixed with alcoholic peptide stock solutions (TFE) to form a solution of CHCl$_3$/alcohol (1/1, v/v) with defined P/L-ratio. Removing the solvents in a nitrogen stream at temperatures above the main phase transition temperature of the respective lipids produced an almost clear lipid/peptide film at the test tube walls. After removing of residual solvent under reduced pressure for 12 h at $T > t_m$, the lipid films were rehydrated with an appropriate amount of buffer solution (20 mM HEPES, 100 mM KCl, 1 mM EDTA, 1 mM DTT, pH 7.4) yielding the desired peptide concentration. After 1 h of incubation at $T > t_m$, the hydrated lipid films were vortexed (30 s) and incubated (5 min at 45 °C) in five cycles. The preparation of the unilamellar vesicles and other peptide/lipid complexes was carried out at temperatures above the transition temperature, i.e. in the lipid fluid $L_\alpha$-phase ($T > t_m$).

3.2 Large Unilamellar Vesicles (LUVs)

The milky MLV suspensions were extruded 31 times through a polycarbonate membrane (100 nm nominal pore size) using a Liposofast mini-extruder (Avestin, Ottawa, Canada) to
produce an almost clear vesicle suspension containing vesicles of 100 nm size with a low degree of polydispersity as determined by dynamic light scattering (see below). \[^4\]

### 4 Spectroscopic and Analytical Methods

#### 4.1 Mass Spectrometry (MS)

Electrospray ionization mass spectra (ESI-MS) were obtained with a *Finnigan* LCQ instrument. High-resolution mass spectra (HRMS-ESI) were obtained with the *Bruker* Apex-Q IV FT-ICR-MS instrument. All synthesized PNA oligomers and PNA/peptide conjugates were characterized by both ESI-MS and HRMS (ESI).

#### 4.2 Dynamic Light Scattering (DLS)

Experimental diffusion coefficients, D, were measured at 25 °C by dynamic light scattering at *Viscotek 802 DLS*. The laser wavelength was 633 nm and the scattering angle was 173°. The Stokes-Einstein relationship: 
\[
D = \frac{k_B T}{3 \pi \eta D_h}
\]
was used to estimate the hydrodynamic radius, \(D_h\) (nm). Here, \(k_B\) is the Boltzman constant and \(\eta\) the solvent viscosity.

#### 4.3 UV-Spectroscopy

Peptide concentrations were estimated with a *Jasco V-550* UV spectrometer (Gross-Umstadt, Germany) while the sample cell was floated with nitrogen. Molecular absorption coefficients were calculated for each oligomer sequence via summation of the monomer absorption coefficients at a certain wavelength. PNA and PNA/peptide conjugate concentrations were estimated at 260 nm (\(\varepsilon_{260\ nm}(C) = 6600\ L\ mol^{-1}\ cm^{-1}\), \(\varepsilon_{260\ nm}(G) = 11700\ L\ mol^{-1}\ cm^{-1}\), \(\varepsilon_{260\ nm}(T) = 8600\ L\ mol^{-1}\ cm^{-1}\), \(\varepsilon_{260\ nm}(A) = 13700\ L\ mol^{-1}\ cm^{-1}\)). Concentrations were calculated with the Lambert-Beer’s law.

#### 4.3.1 UV-Melting Analysis

UV-melting curves were recorded at the same UV spectrometer as mentioned above by using a *Jasco ETC-505S/ETC-505T* peltier temperature controller. All measurements were carried out in a micro quartz glass cell of 1 cm path length in a HEPES buffer solution (20 mM HEPES, 100 mM NaCl, 1mM EDTA, pH 7.4) at a concentration of 4 µM for each oligomer of the PNA duplex. Data collections were carried out at 260 nm with a heating rate of 0.4 °C min\(^{-1}\). The protocol for the melting curve recordings is as follows: 20 °C → 80 °C (10 min), 80 °C (3 min), 80 °C → -2 °C (26 min) → 0 °C (15 min) → 85 °C (180 min) → -2 °C (195
min) $\rightarrow$ 85 °C (180 min) $\rightarrow$ -2 °C (180 min) $\rightarrow$ 28 °C (5 min). After data collection for both, heating and cooling cycles, the hyperchromicity ($H$) was calculated according to the equation: $H(\%) = 100 \times (A(T) - A_0) / A_0$, where $A(T)$ is the absorbance given at any temperature and $A_0$ is the minimum absorbance. The melting temperature $T_m$ was estimated at the inflection point of the curve.

**Figure S1.** Melting curves of PNAs and PNA-linker constructs employed. a) Parallel orientation of PNA1/PNA3 (red curve) and PNA1-SybLinker/PNA3-SxLinker (orange curve); b) Antiparallel orientation of PNA1/PNA2 (green curve) and PNA1-SybLinker/PNA2-SxLinker (blue curve). c) Respective PNA and PNA-linker sequences.

### 4.4 Fluorescence Spectroscopy

Fluorescence spectra were obtained at a *Jasco FP 5600* (Gross-Umstadt, Germany) under temperature control using a thermostat (Model 1162A, *VWR International*, Darmstadt, Germany). The excitation and emission bandwidth was set to 5 nm, the data pitch was 1 nm and the response time was adjusted to 0.1 s. NBD-DOPE was used as fluorescence donor, rhodamine-DOPE as acceptor. Data were recorded at a P/L-ratio of 1/200 in DOPC/DOPE/cholesterol (50/25/25) LUVs.

#### 4.4.1 Lipid Mixing Assay

Lipid mixing assay excitation was performed at 460 nm with a detection of the fluorescence emission at 534 nm.$^{[5]}$ NBD/rhodamine labeled vesicles (1.5 % NBD-DOPE and 1.5 % rhodamine-DOPE), containing PNA1-peptide constructs (PNA1-SybTMD or PNA1-SxTMD), were mixed with unlabeled vesicles, containing PNA-SxTMD constructs (PNA1-...
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SxTMD, PNA2-SxTMD or PNA3-SxTMD) at a defined ratio (1 to 4); the total lipid concentration in cuvette was 180 µM. The reaction buffer was 20mM HEPES, 100 mM KCl, 1 mM EDTA, 1 mM DTT pH 7.4 and the reaction temperature was 25 ºC. The change in donor intensity is plotted as:

\[ F(\%) = 100*(F_t - F_0)/(F_{total} - F_0) \]

with \( F_0 \) being the donor intensity at \( t = 0 \) before lipid mixing and \( F_{total} \) the donor intensity after disruption of the vesicles in 1 % (w/v) Triton X-100. The inner leaflet mixing assay was exactly identical, except for the fact, that the NBD/rhodamine- labeled vesicles were treated with sodium dithionite as described below.

4.4.2 Reduction of NBD/Rhodamine Labeled Vesicles
A 1:1 mixture of NBD/rhodamine labeled vesicles (5 mM) and 50 mM \( \text{Na}_2\text{S}_2\text{O}_4 \) (20 mM HEPES, pH 7.4) was incubated at room temperature for 5 min. Free sodium dithionite was removed by gel filtration using Superdex G-50 Superfine columns (GE Healthcare). Reduction was immediately carried out before use of the vesicles.[6]

4.4.3 Content Mixing Assay
A vesicle population with an encapsulated fluorophore (Sulforhodamine B) is mixed with vesicles without Sulforhodamine B. The observed increasing of fluorescence caused by decreasing of fluorophore concentration indicates that vesicle contents are mixed, that is fusion takes place.

Content mixing assay excitation was performed at 490 nm with a detection of the fluorescence emission at 567 nm.[7] The vesicles, containing PNA1-SybTMD constructs with encapsulated sulforhodamine B (20 mM in 20 mM HEPES, 100 mM KCl, 1 mM EDTA, 1 mM DTT, pH 7.4) were extruded through a polycarbonate membrane (100 nm nominal pore size), after that, the free sulforhodamine B (SRB) was removed by gel filtration using Superdex G-50 Superfine columns (GE Healthcare). Unlabeled vesicles, containing PNA-SxTMD constructs (PNA1-SxTMD, PNA2-SxTMD or PNA3-SxTMD), were prepared in the same way as described in section 3.2. Both types of vesicles were mixed at a defined ratio (1:4); the total lipid concentration in the cuvette was 180 µM. The reaction buffer was 20mM HEPES, 100 mM KCl, 1 mM EDTA, 1 mM DTT, pH 7.4 and the reaction temperature was 25 ºC. The change in SRB intensity is plotted as:

\[ F(\%) = 100*(F_t - F_0)/(F_{total} - F_0) \]
with $F_0$ being the SRB intensity at $t = 0$ before content mixing and $F_{\text{total}}$ the SRB intensity after disruption of the vesicles in 1% (w/v) Triton X-100.

5 Synthesis of Peptides, PNAs and PNA/Peptide Conjugates

5.1 Synthesis of Peptides

Linear peptides (SxLinker, SxTMD, SybLinker, SybTMD) were synthesized by SPPS on the CEM Liberty device as described above. Peptide acids were synthesized at Wang resins with loading densities 0.2-0.5 mmol·g$^{-1}$.

**SxLinker**

\[
\text{NH}_2-\text{YQSKARRKIMG-COOH}
\]

\[
C_{63}H_{112}N_{22}O_{16}S \text{ [1465.80]}
\]

ESI-MS: $m/z = 489.29$ [M+3H]$^{3+}$, 733.42 [M+2H]$^{2+}$, 1465.85 [M].

HR-MS: $C_{63}H_{113}N_{22}O_{16}S$ [M+3H]$^{3+}$ calc. 489.28552, found 489.28557.

**SxTMD**

\[
\text{NH}_2-\text{YQSKARRKIKIMICCVILGISASTIGGIFG-COOH}
\]

\[
C_{165}H_{288}N_{44}O_{38}S_3 \text{ [3479.04]}
\]

ESI-MS: $m/z = 696.82$ [M+5H]$^{5+}$, 870.77 [M+4H]$^{4+}$, 1060.68 [M+3H]$^{3+}$.

HR-MS: $C_{159}H_{281}N_{42}O_{38}S_3$ [M+5H]$^{5+}$ calc. 696.6096, found 696.6110; $C_{159}H_{280}N_{42}O_{38}S_3$ [M+4H]$^{4+}$ calc. 870.5102, found 870.5119.

**SybLinker**

\[
\text{NH}_2-KRKYWKKNLKMMG-COOH
\]

\[
C_{83}H_{132}N_{23}O_{16}S_2 \text{ [1769.23]}
\]

ESI-MS: $m/z = 590.32$ [M+3H]$^{3+}$, 884.98 [M+2H]$^{2+}$, 1768.96 [M].

HR-MS: $C_{83}H_{131}N_{23}O_{16}S_2$ [M+3H]$^{3+}$ calc. 590.32194, found 590.32160.

**SybTMD**

\[
\text{NH}_2-KRKYWKKNLKMIMIGVIČAILILIIIVYFS-T-COOH
\]

\[
C_{190}H_{309}N_{42}O_{30}S_3 \text{ [3885.02]}
\]
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**ESI-MS**: \( m/z = 777.67 \ [M+5H]^5^+ \), 971.83 \ [M+4H]^4^+ \), 1295.43 \ [M+3H]^3^+ \.

**HR-MS**: \( C_{190}H_{313}N_{42}O_{38}S_3 \) \ [M+5H]^5^+ \) calc. 777.4597, found 777.4608; \( C_{190}H_{312}N_{42}O_{38}S_3 \) \ [M+4H]^4^+ \) calc. 971.5728, found 971.5742.

5.2 Synthesis of PNAs and Short PNA/Peptide Conjugates
PNAs were manually synthesized on Wang resins according to methods described above in section 2.4. The peptide backbones of PNA6Linker constructs were synthesized with the CEM liberty MW-peptide synthesizer at 0.1 mmol scale (see section 2.3). The loading densities of all resins were low and varied between 0.2-0.5 mmol g\(^{-1}\). Wang resin cleavage was performed and followed by HPLC.

**PNA 1**

\[
C_{110}H_{138}N_{59}O_{32} \ [2784.70]
\]

**HPLC** (**Phenomenex Jupiter** column, RP-C18, 250 × 10.0 mm, 5 µm, 300 Å, gradient: 0-25 % B2 in 25 min): \( t_R = 23.46 \) min.

**ESI-MS**: \( m/z = 696.79 \ [M+4H]^4^+ \), 928.71 \ [M+3H]^3^+ \), 1392.56 \ [M+2H]^2^+ \.

**HR-MS**: \( C_{110}H_{141}N_{59}O_{32} \) \ [M+3H]^3^+ \) calc. 928.7057, found 928.7059; \( C_{110}H_{140}N_{59}O_{32} \) \ [M+2H]^2^+ \) calc. 1392.5550, found 1392.5561

**PNA 2**

\[
C_{110}H_{138}N_{59}O_{32} \ [2784.70]
\]

**HPLC** (**Phenomenex Jupiter** column, RP-C18, 250 × 10.0 mm, 5 µm, 300 Å, gradient: 0-25 % B2 in 25 min): \( t_R = 23.33 \) min.

**ESI-MS**: \( m/z = 696.79 \ [M+4H]^4^+ \), 928.71 \ [M+3H]^3^+ \), 1392.56 \ [M+2H]^2^+ \.)
**Supporting Information**

**HR-MS:** \( \text{C}_{110}\text{H}_{141}\text{N}_{59}\text{O}_{32} \) [M+3H]\(^3+\) calc. 928.7057, found 928.7060; \( \text{C}_{110}\text{H}_{140}\text{N}_{58}\text{O}_{32} \) [M+2H]\(^2+\) calc. 1392.5550, found 1392.5556

**PNA 3**

\[
\text{C}_{110}\text{H}_{138}\text{N}_{58}\text{O}_{32} [2784.70]
\]

**HPLC** (Phenomenex Jupiter column, RP-C18, 250 × 10.0 mm, 5 µm, 300 Å, gradient: 0-25 % B2 in 25 min): \( t_R = 23.86 \) min.

**ESI-MS:** \( m/z = 696.79 \) [M+4H]\(^4+\), 928.71 [M+3H]\(^3+\), 1392.56 [M+2H]\(^2+\).

**HR-MS:** \( \text{C}_{110}\text{H}_{142}\text{N}_{59}\text{O}_{32} \) [M+4H]\(^4+\) calc. 696.7811, found 696.7815; \( \text{C}_{110}\text{H}_{141}\text{N}_{58}\text{O}_{32} \) [M+3H]\(^3+\) calc. 928.7057, found 928.7059; \( \text{C}_{110}\text{H}_{140}\text{N}_{58}\text{O}_{32} \) [M+2H]\(^2+\) calc. 1392.5550, found 1392.5549

**PNA1-SybLinker**

\[
\text{C}_{191}\text{H}_{262}\text{N}_{80}\text{O}_{46}\text{S}_2 [4478.85]
\]

**HPLC** (Phenomenex Jupiter column, RP-C18, 250 × 10.0 mm, 5 µm, 300 Å, gradient: 20-50 % B2 in 25 min): \( t_R = 18.76 \) min.

**ESI-MS:** \( m/z = 640.59 \) [M+7H]\(^7+\), 747.18 [M+6H]\(^6+\), 896.41 [M+5H]\(^5+\), 1120.26 [M+4H]\(^4+\), 1493.34 [M+3H]\(^3+\).

**HR-MS:** \( \text{C}_{191}\text{H}_{268}\text{N}_{80}\text{O}_{46}\text{S}_2 \) [M+7H]\(^7+\) calc. 640.4367, found 640.4378; \( \text{C}_{191}\text{H}_{268}\text{N}_{80}\text{O}_{46}\text{S}_2 \) [M+6H]\(^6+\) calc. 747.0083, found 747.0092.
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**PNA2-SxLinker**

\[
\text{C}_{177}\text{H}_{257}\text{N}_{81}\text{O}_{47}\text{S} \ [4303.60]
\]

**HPLC** (*Phenomenex Jupiter* column, RP-C18, 250 × 10.0 mm, 5 µm, 300 Å, gradient: 10-40 % B2 in 20 min): \( t_R = 16.0 \) min.

**ESI-MS:** \( m/z = 615.58 \) [M+7H]\(^{7+}\), 718.01 [M+6H]\(^{6+}\), 861.41 [M+5H]\(^{5+}\), 1076.51 [M+4H]\(^{4+}\), 1435.00 [M+3H]\(^{3+}\).

**HR-MS:** C\(_{177}\)H\(_{263}\)N\(_{81}\)O\(_{47}\)S [M+6H]\(^{6+}\) calc. 717.8395, found 717.8402; C\(_{177}\)H\(_{262}\)N\(_{81}\)O\(_{47}\)S [M+5H]\(^{5+}\) calc. 861.2059, found 861.2058.

**PNA3-SxLinker**

\[
\text{C}_{177}\text{H}_{257}\text{N}_{81}\text{O}_{47}\text{S} \ [4303.60]
\]

**HPLC** (*Phenomenex Jupiter* column, RP-C18, 250 × 10.0 mm, 5 µm, 300 Å, gradient: 10-40 % B2 in 20 min): \( t_R = 15.76 \) min.

**ESI-MS:** \( m/z = 615.58 \) [M+7H]\(^{7+}\), 718.01 [M+6H]\(^{6+}\), 861.41 [M+5H]\(^{5+}\), 1076.51 [M+4H]\(^{4+}\), 1435.00 [M+3H]\(^{3+}\).

**HR-MS:** C\(_{177}\)H\(_{263}\)N\(_{81}\)O\(_{47}\)S [M+6H]\(^{6+}\) calc. 717.8395, found 717.8402; C\(_{177}\)H\(_{262}\)N\(_{81}\)O\(_{47}\)S [M+5H]\(^{5+}\) calc. 861.2059, found 861.2057.

### 5.3 Synthesis of PNA/Peptide Conjugates

Peptide backbones of PNA/peptide constructs were synthesized with the *CEM liberty* MW-peptide synthesizer using *Wang* resin (loading: 0.2-0.5 mmol·g\(^{-1}\)) at 0.1 mmol scale. PNA attachment was performed via manual PNA/peptide conjugate synthesis (see section 2.4). *Wang* resin cleavage was performed and followed by HPLC.
PNA1-SybTMD

HPLC (Phenomenex Jupiter column, RP-C4, 250 × 10.0 mm, 5 µm, 300 Å, gradient: 5-100 % B1 in 30 min): \( t_R = 28.1 \) min.

ESI-MS: \( m/z = 942.92 [M+7H]^{7+}, 1099.90 [M+6H]^{6+}, 1319.48 [M+5H]^{5+} \)

HR-MS: \( C_{298}H_{447}N_{99}O_{69}S_3 [M+6H]^{6+} \) calc. 1099.3949, found 1099.3956; \( C_{298}H_{446}N_{98}O_{68}S_3 [M+5H]^{5+} \) calc. 1319.0724, found 1319.0726.

PNA1-SxTMD

HPLC (Phenomenex Jupiter column, RP-C4, 250 × 10.0 mm, 5 µm, 300 Å, gradient: 5-100 % B1 in 30 min): \( t_R = 24.7 \) min.

ESI-MS: \( m/z = 903.33 [M+7H]^{7+}, 1053.54 [M+6H]^{6+}, 1264.25 [M+5H]^{5+} \)

HR-MS: \( C_{273}H_{428}N_{101}O_{69}S_3 [M+7H]^{7+} \) calc. 903.0316, found 903.0344; \( C_{273}H_{427}N_{100}O_{68}S_3 [M+6H]^{6+} \) calc. 1053.3690, found 1053.3677; \( C_{273}H_{426}N_{101}O_{69}S_3 [M+5H]^{5+} \) calc. 1263.8413, found 1263.8387.

PNA2-SxTMD

HPLC (Phenomenex Jupiter column, RP-C4, 250 × 10.0 mm, 5 µm, 300 Å, gradient: 5-100 % B1 in 30 min): \( t_R = 24.5 \) min.

ESI-MS: \( m/z = 903.33 [M+7H]^{7+}, 1053.71 [M+6H]^{6+}, 1264.25 [M+5H]^{5+} \).
Supporting Information

**HR-MS:** $C_{273}H_{428}N_{101}O_{69}S_3\ [M+7H]^7^+$ calc. 903.1748, found 903.1743; $C_{273}H_{427}N_{101}O_{69}S_3\ [M+6H]^6^+$ calc. 1053.3690, found 1053.3698; $C_{273}H_{426}N_{101}O_{69}S_3\ [M+5H]^5^+$ calc. 1264.0418, found 1264.0393.

**PNA3-SxTMD**

\[
\begin{array}{c}
\text{C}_{273}\text{H}_{427}\text{N}_{101}\text{O}_{69}\text{S}_{3}\ [6318.24]
\end{array}
\]

**HPLC** (Phenomenex Jupiter column, RP-C4, 250 × 10.0 mm, 5 µm, 300 Å, gradient: 5-100 % B1 in 30 min): $t_R = 25.2$ min.

**ESI-MS:** $m/z = 903.33\ [M+7H]^7^+$, 1053.71 [M+6H]$^6^+$, 1264.25 [M+5H]$^5^+$.

**HR-MS:** $C_{273}H_{428}N_{101}O_{69}S_3\ [M+7H]^7^+$ calc. 903.0316, found 903.0337; $C_{273}H_{427}N_{101}O_{69}S_3\ [M+6H]^6^+$ calc. 1053.3690, found 1053.3690; $C_{273}H_{426}N_{101}O_{69}S_3\ [M+5H]^5^+$ calc. 1264.0418, found 1264.0395.

**References**


