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Coiled Coil Peptides and Polymer–Peptide Conjugates: Synthesis, Self-Assembly, Characterization and Potential in Drug Delivery Systems

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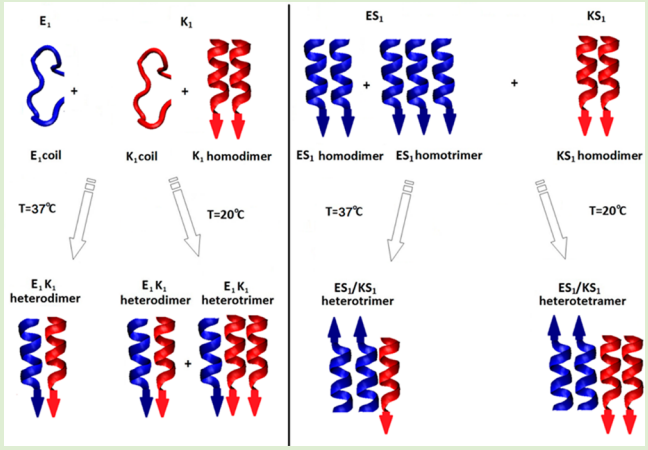
ABSTRACT: Coiled coils are a common structural motif in many natural proteins that can also be utilized in the design and preparation of drug delivery systems for the noncovalent connection of two macromolecules. In this work, two different pairs of peptides forming coiled coil hetero-oligomers were designed, synthesized, and characterized. While the peptide sequences $(VAALEKE)_4$ and $(VAALKEK)_4$ predominantly form coiled coil heterodimers with randomly orientated peptide chains, $(IAALESE)_2$-IAALESKIAALESE and IAALKSKIAALKSE-$(IAALKSK)_2$ tend to form higher heterooligomers with an antiparallel orientation of their peptide chains. The associative behavior of these peptides was studied in aqueous solutions using circular dichroism spectroscopy, size-exclusion chromatography, isothermal titration calorimetry and sedimentation analyses. The orientation of the peptide chains in the coiled coil heterodimers was assessed using fluorescence spectroscopy with fluorescence resonance energy transfer labels attached to the ends of the peptides. The formation of the heterodimer can be used as a general method for the selective noncovalent conjugation of a specific targeting moiety with various drug carrier systems; this process involves simple self-assembly in a physiological solution before drug administration. The preparation of targeted macromolecular therapeutics consisting of a synthetic polymer drug carrier and a recombinant protein targeting ligand is discussed.



INTRODUCTION

Coiled coils are folding motifs present in many natural proteins. They consist of two to six right-handed alpha helices forming a left-handed superhelical bundle. The amino acid sequence of the coiled coil peptides typically contains repetitions of seven amino acid residues (heptad repeats) denoted $(a\text{-}b\text{-}c\text{-}d\text{-}e\text{-}f\text{-}g)_n$. The *a* and *d* positions are occupied by hydrophobic residues, while the *e* and *g* positions are usually occupied by charged or hydrophilic residues. The rules governing the formation of coiled coils and their properties were thoroughly investigated; the results of these investigations have been summarized in numerous review articles.[1−4] Coiled coils have become attractive for investigators in the field of drug delivery in addition to those studying protein structures and interactions.[5−12]

We have recently reported a coiled coil heterodimer consisting of $(VAALEKE)_4$ and $(VAALKEK)_4$ sequences. The heterodimer was used as a linker attaching two different recombinant targeting protein ligands to synthetic polymer drug carriers based on *N*-(2-hydroxypropyl)methacrylamide (HPMA) copolymers. In one case, a single chain antibody fragment (scFv) of monoclonal antibody M75 (attached via the coiled coil) successfully targeted an HPMA copolymer to the corresponding antigen carbonic anhydrase IX.[13] In another study, scFv of B1 monoclonal antibody was shown to target HPMA copolymers bearing doxorubicin to BCL1 leukemia cells.[14] The macromolecular chains exerted no observable steric hindrance preventing the formation of the coiled coil structure. The cytotoxicity of the polymer–doxorubicin conjugate against BCL1 cells increased approximately 100-fold after adding the targeting ligand; no difference was observed between the cytotoxicity of the targeted and nontargeted polymer-

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doxorubicin conjugates against a negative control cell line, 38C13.

Several parameters are important when applying a coiled coil heterodimer as a linker between a polymer and a recombinant protein or, generally, between two macromolecules. The coiled coil heterodimer must remain sufficiently stable under physiological conditions: the binding constant of the interaction between the helices should be high enough to prevent spontaneous dissociation of the heterodimer. The mutual orientation of the peptide chains (parallel versus antiparallel) might influence the properties of the resulting complex. The antiparallel orientation of the helices should be favorable due to the steric demands of the targeting ligand (recombinant protein) and the polymer to which it should be attached (Figure 1).

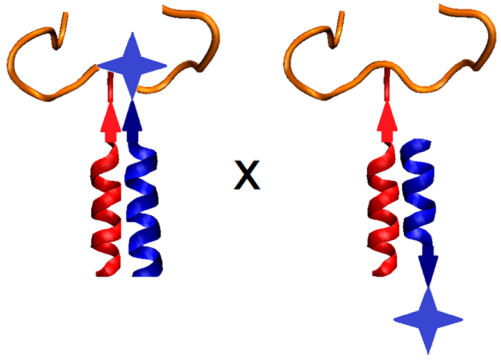

Figure 1. Schematic representation of a polymer drug carrier bearing a protein targeting ligand attached via parallel (left) and antiparallel (right) coiled coil heterodimers.

In this work, we describe the physicochemical properties and associative behavior of two pairs of coiled coil heterodimers. The first coiled coil heterodimer consists of the peptides $(VAALEKE)_4$ and $(VAALKEK)_4$, enabling both parallel and antiparallel orientation of the peptide chains. The other coiled coil heterodimers $(IAALESE)_2$-IAALESKIAALESE and IAALKSKIAALKSE-$(IAALKSK)_2$ were already designed to prefer an antiparallel orientation of the peptides due to the electrostatic interactions between the amino acid residues. We confirmed the mutual orientation of the peptide in the coiled coil heterodimers using fluorescence resonance energy transfer (FRET) labels attached to the ends of the peptides. The associative behavior of the peptides in aqueous solutions was further investigated by circular dichroism (CD) spectroscopy, size-exclusion chromatography (SEC), isothermal titration calorimetry (ITC) and sedimentation analysis in analytical ultracentrifuge (AUC). In addition to the above-described peptides, model diblock copolymers consisting of a semitelechelic poly(HPMA) block and a coiled coil peptide block were also synthesized and characterized. Their properties were studied while focusing on the conjugation of the two macromolecular poly(HPMA) chains via a coiled coil peptide structure.

Considering the intended future application of the coiled coil heterodimers for attachment of biomacromolecules (e.g., recombinant proteins) to polymer carriers of drugs, the synthetic polymer poly(HPMA) serves in this study as model of the biologically active macromolecule. Besides the above-mentioned physicochemical properties of the coiled coil associates the biologically relevant properties (e.g., biodegradability or immunogenicity) of these systems under physiological conditions are also important. We already know that the synthetic hydrophilic polymers (pHPMA or poly(ethylene glycol) (PEG)) effectively shield the proteins in the polymer conjugates against the immune system components and substantially reduce the immunogenicity and also slow down the proteolytic degradation of the proteins.[15] These biological properties will be the subject of our future investigations.

EXPERIMENTAL SECTION

Materials and Methods. Piperidine, ethyldiisopropylamine (DIPEA), trifluoroacetic acid (TFA), 1-hydroxybenzotriazole (HOBt), triisopropylsilane (TIPS), *N*,*N*-dimethylformamide (DMF) and dimethyl sulfoxide (DMSO) were purchased from Sigma-Aldrich, Prague, Czech Republic. All fluorenylmethyloxycarbonyl-amino acids (Fmoc-aa) were of L-configuration. 2-Chlorotrityl chloride resin, TentaGel Rink amide resin, (benzotriazol-1-yloxy)-trispyrrolidinophosphoniumhexafluorophosphate (PyBOP) protected amino acid derivatives, and Fmoc-aminoethyltetraethylene glycol ($Fmoc\text{-}Peg_4$) were purchased from Iris Biotech, Marktredwitz, Germany.

(2S)-2-(9H-fluoren-9-ylmethoxycarbonylamino)-5-oxo-5-[2-[(5-sulfo-1-naphthyl)amino]-ethylamino]pentanoic acid (Fmoc-Glu-(Edans)) and (2S)-6-[[4-[(E)-[4-(dimethylamino)-phenyl]azo]-benzoyl]amino]-2-(9H-fluoren-9-ylmethoxycarbonylamino)hexanoic acid (Fmoc-Lys(Dabcyl) were purchased from Merck, Prague, Czech Republic.

All other reagents and solvents were purchased from Sigma-Aldrich, Prague, Czech Republic. All chemicals and solvents were analytical grade. Solvents were purified and dried using standard procedures.

The peptides were characterized before and after purification using a reversed phase high pressure liquid chromatography (HPLC) equipped with a Chromolith RP18-e 100−4.6 mm column (Merck, Prague, Czech Republic), with a 0−100% gradient of acetonitrile in water containing 0.1% TFA while using a UV−vis photodiode array detector (Shimadzu, Japan). All crude peptides were purified using a reversed phase HPLC on a ChromolithSemiPrep RP18-e, 100−10 mm (Merck, Germany) while using a 30−35% gradient of acetonitrile in water containing 0.1% TFA over 10 min at 5 mL/min. Matrix assisted laser desorption ionization time-of-flight (MALDI-TOF) spectrometry was carried out using a BrukerBiflex III mass spectrometer. The peptide loading of the resin was determined by measuring the amount of Fmoc group (0.1 mmol/g) released with 25% piperidine in DMF spectrophotometrically.[16]

The emission spectra were measured using a FP-6200 spectrofluorometer (Jasco Europe, Italy) in 0.05 M phosphate buffer with 0.15 M NaCl, pH 7.4 (PBS). The measurement range spanned 400 to 600 nm, and the excitation wavelength was set to 335 nm. The concentration of the peptides was 0.1 mg/mL, and the spectra were measured in 0.5 mL cuvettes. The content of terminal aza-dibenzocyclooctyne (DBCO) groups in the semitelechelic polymers was determined spectrophotometrically with a Helios Alpha UV/vis spectrophotometer (Thermo Spectronic, UK) using the absorption coefficient for DBCO in methanol: ε_{292} = 13 000 L mol^{-1} cm^{-1}.

Isothermal Titration Calorimetry. Microcalorimetry was carried out using a MicroCal iTC200 isothermal titration calorimeter. The experiment involved a consecutive injection of the concentrated PBS solution of peptide ES_1 or E_1 into a calorimeter cell containing 280 μL of the PBS solution of KS_1 or K_1. The concentrations of the peptide solutions were chosen assuming a 1:1 stoichiometric binding between the compounds. The KS_1 solution was added using a 40 μL injection syringe, with the tip modified to act as a stirrer. The stirring speed was set to 1000 rpm. The injection volume and the time between the injections were 1 μL and 3 min, respectively. The measurements were conducted in the range of 15−50 °C. The data were analyzed using NITPIC software.[17] The experimental enthalpy was obtained by the integration of the raw data signal. The integrated molar enthalpy change per injection was obtained by dividing the experimental enthalpy by the number of moles of the component in the syringe.

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The final enthalpograms plot the integral molar enthalpy as a function of the molar ratio of the ES_1 to KS_1 (E_1/K_1) solutions.

To evaluate the binding process further, the "two sets of sites" fitting model was primarily used:

$$K_1 = \frac{\theta_1}{(1-\theta_1)[X]}; \quad K_2 = \frac{\theta_2}{(1-\theta_2)[X]}$$

$$X_t = [X] + M_t(n_1\theta_1 + n_2\theta_2)$$

where K_1 and K_2 are the interaction binding constants, Θ_1, Θ_2 are the fractions occupied by the peptide, n_1, and n_2 indicate the stoichiometry of the process, $[X]$ is the free concentration of the second peptide, and X_t is the bulk concentration of the second peptide (the peptide that was added from the syringe to the cell). The model allows the stoichiometry of the binding process to be calculated, as well as the binding constant and the enthalpy/entropy contribution values. For some selected temperatures with one distinguished processes, the "one set of sites" model was used.

CD Spectroscopy. The CD measurements were performed using Jasco-815 dichrograph, as described previously.[13] Briefly, the peptide spectra were measured in a 1 mm quartz cell from 190 to 260 nm, scanning at 20 nm/min with a response time of 4 s and using two computer-averaged accumulations. The CD spectra of the HPMA copolymers formed by poly(HPMA) blocks connected by a coiled coil linker (PE_1/ PK_1 and PES_1/PKS_1) were measured in 1 mm quartz cells from 190 to 260 nm, scanning at 5 nm/min with a response time of 32 s and four computer-averaged accumulations. The sample concentration was maintained at 0.2 mg/mL in 0.05 M phosphate buffer with 0.15 M NaCl, pH 7.4 (PBS). The temperature-dependent measurements were performed using a PTC-423S/L Peltier-type temperature control system in a 1 cm quartz cell, allowing the measured solution to be stirred. The measurement to determine the α-helical content was performed at 222 nm (temperature interval 2−95 °C, temperature gradient of 25 °C per h, response time of 32 s).

The spectra were expressed as the mean molar ellipticity at 222 nm per residue and were converted into the α-helical fraction (f_H) using a two-state model.[18]

Size-Exclusion Chromatography. The molecular weights and polydispersities of the polymers and copolymers were measured by SEC using an HPLC system (Shimadzu, Japan) equipped with refractive index (RI), UV and multiangle light scattering (MALS) DAWN 8 EOS detectors (Wyatt Technology, USA), a Superose 6 column (GE Healthcare, USA), and 0.3 M acetate buffer (pH 6.5) as an eluent at 0.5 mL min^{-1} or a MicroSuperose 6 column in 0.05 M phosphate buffer with 0.15 M NaCl (pH 7.4 (PBS)) at 0.1 mL min^{-1}. The molecular weights were calculated from the light-scattering detector based on the known injected mass while assuming 100% mass recovery. For poly(HPMA) homopolymer, a dn/dc value of 0.167 mL/g was used for the molecular weight calculations. The characterization of the peptides and the formation of the coiled coil heterodimers were monitored using SEC with a Superdex Peptide column (GE Healthcare, USA) in PBS at 0.5 mL min^{-1}.

Analytical Ultracentrifugation. The sedimentation analysis was performed as described previously[13] using a ProteomeLab XL-I analytical ultracentrifuge equipped with an An50Ti rotor (BeckmanCoulter, USA) at a 0.7 or 0.233 mg/mL total loading concentration in 0.05 M sodium phosphate and 0.15 M NaCl buffer (pH 7.4 (PBS)), which was also used as a reference. The sedimentation velocity experiments were carried out at 50000 rpm and 20 °C; the absorbance scans were recorded at 230 nm in 10 min intervals, with 30 μm spatial resolution. The sedimentation equilibrium experiments were carried out at 24−28−32−36−40−44−48000 rpm and 20 °C, while the absorbance scans were recorded at 230 nm in 18 h intervals, with a 10 μm spatial resolution. The buffer density and peptide partial specific volumes were estimated in SEDNTERP 1.09 (www.jphilo.mailway.com). The partial specific volume of the Peg was estimated to be 0.83 mL g^{-1} at 20 °C, and the partial specific volumes of the peptides with Peg_4 spacers were then calculated based on the molar ratio of their peptide and Peg components. The data were analyzed with SEDFIT 14.1 and SEDPHAT 10.58d.[19,20]

Synthesis of peptides E_1, K_1, ES_1, and KS_1. The linear peptides E_1 and K_1, consisting of four repeating heptads (VAALEKE or VAALKEK) terminated with an azide group, were prepared using solid-phase fragment condensation of fully protected heptapeptides, as described previously.[13] Peptides ES_1 and KS_1 (S stands for serine in the corresponding peptide sequences) were prepared analogously, using solid phase fragment condensation of the fully protected heptapeptides IAALESE, IAALESK, IAALKSE, and IAALKSK in the appropriate order. The protected heptapeptides were prepared using standard Fmoc procedures with PyBOP (2.5 equiv), HOBt (2.5 equiv) and DIPEA (5.0 equiv) in DMF. The pseudoproline dipeptides Fmoc-Lys(Boc)-Ser($\psi^{Me,Me}$Pro)−OH and Fmoc-Glu(OtBu)-Ser-($\psi^{Me,Me}$Pro)−OH were used in the appropriate condensation step. The peptides were purified and characterized using HPLC and MALDI-TOF MS (Table 1).

Table 1. Molecular Weights of the Coiled Coil Peptides

sample	structure	theoretical mol. wt.[a]	measured mol. wt.[b]
E_1	5-azidopentanoyl-Peg_4-(VAALEKE)$_4$	3350.9	3351.8
K_1	5-azidopentanoyl-Peg_4-(VAALKEK)$_4$	3347.1	3347.7
ES_1	5-azidopentanoyl-Peg_4-(IAALESE)$_2$-IAALESKIAALESE	3241.7	3242.7
KS_1	5-azidopentanoyl-Peg_4-IAALKSKIAALKSE (IAALKSK)$_2$	3236.0	3237.5

[a]Calculated monoisotopic molecular weight of the peptide acid. [b]M +H relative molecular weight values from the MALDI-TOF analysis.

Synthesis of Peptides E_2 and ES_2. Peptide E_2 was synthesized by the solid-phase fragment condensation of fully protected heptapeptides (VAALEKE), while peptide ES_2 was prepared analogously by the solid-phase fragment condensation of the fully protected heptapeptides IAALESE and IAALESK in the appropriate order. After condensing the fourth heptad, the Fmoc protecting group was removed using 25% piperidine in DMF. Condensation of Fmoc-Glu(Edans) was carried out using standard Fmoc procedures: adding the Fmoc-Glu(Edans) (2.5 equiv), PyBOP (2.5 equiv), HOBt (2.5 equiv), and DIPEA (5.0 equiv) in DMF. After removing the Fmoc group and performing an acetylation, the peptide was cleaved from the resin using 95% TFA, 2.5% TIPS and 2.5% H_2O. A similar synthesis was used for peptides K_2 and KS_2, which were prepared using a fragment condensation of the fully protected heptapeptides VAALKEK or IAALKSK and IAALKSE, in the appropriate order. The condensation and cleavage of Fmoc-Lys(Dabcyl) were performed using the same conditions as E_2 or ES_2. After purification, the peptides were characterized using HPLC and MALDI-TOF MS (Table 2).

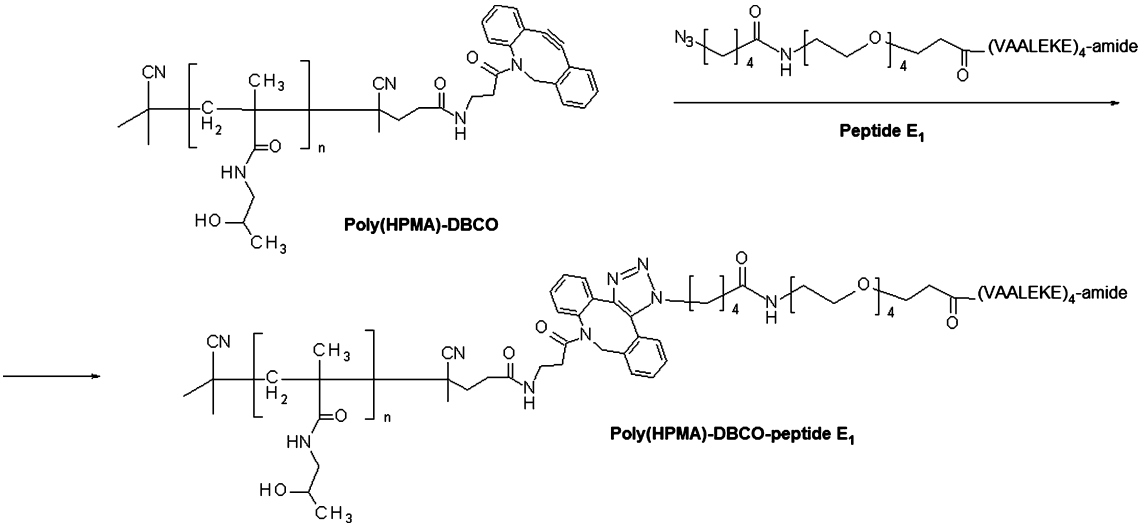
Synthesis of Peptides K_3 and KS_3. TentaGel Rink amide resin (0.1 g, 0.01 mmol of Fmoc groups) was deprotected with 25%

Table 2. Molecular Weights of the Peptides Labeled with FRET Dyes

sample	structure	theoretical mol. wt.[a]	measured mol. wt.[b]
E_2	Ac-(γ-Edans)E(VAALEKE)$_4$	3397.8	3399.2
K_2	Ac-(ε-Dabcyl)K(VAALKEK)$_4$	3396.1	3397.4
K_3	Ac-(VAALKEK)$_4$-(ε-Dabcyl)K	3396.1	3396.8
ES_2	Ac-(γ-Edans)E-(IAALESE)$_2$-IAALESKIAALESE	3289.6	3289.6
KS_2	Ac-(ε-Dabcyl)K-IAALKSKIAALKSE (IAALKSK)$_2$	3285.1	3286.4
KS_3	Ac-IAALKSKIAALKSE (IAALKSK)$_2$-(ε-Dabcyl)K	3285.0	3286.5

[a]Calculated monoisotopic molecular weights of the peptide acid. [b]M +H relative molecular weight values from the MALDI-TOF analysis.

Scheme 1



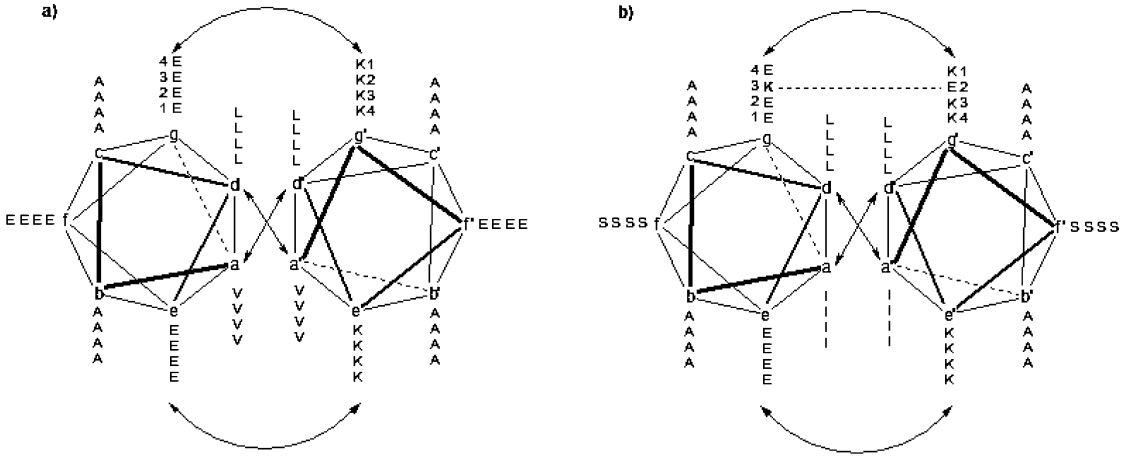


Figure 2. Helical wheel diagrams of the antiparallel coiled coil heterodimers E_1/K_1 (a) and ES_1/KS_1 (b).

piperidine in DMF and washed with DMF. Fmoc-Lys(Dabcyl) was attached to the resin using standard Fmoc procedures, as described above. The peptides K_3 and KS_3 were assembled by the solid phase fragment condensation of the fully protected heptapeptides VAALKEK or IAALKSK and IAALKSE. The peptides were purified and characterized using HPLC and MALDI-TOF MS (Table 2).

Synthesis of Poly(HPMA)−Peptide Polymer Conjugates PE_1, PK_1, PES_1, and PKS_1. The polymer−peptide diblock copolymers were prepared as described previously.[21] Briefly, a peptide azide (0.7 mg, 0.2 mmol) was attached to the semitelechelic polymer with a terminal DBCO group (poly(HPMA)−DBCO, Scheme 1) (6.5 mg, 0.2 mmol of DBCO) in 120 mL of water. The progress of the coupling reaction was monitored by HPLC. After 1 h, the product was purified using semipreparative ChromolithSemiPrep RP-18e columns, (Merck, Germany) with a linear gradient of water−acetonitrile (0−100% acetonitrile) and 0.1% TFA (a peak from 7.5 to 10 min, UV detection at 292 nm) before being freeze-dried, typically yielding 6.2 mg of polymer conjugates.

RESULTS AND DISCUSSION

Design and Synthesis of the Peptides. The primary structures of the coiled coil forming peptides were designed according to the general rules governing the formation and properties of coiled coil heterodimers. Helical wheel diagrams (Figure 2) were used to design the sequences forming the coiled coil peptides.

Both coiled coil heterodimers consist of repeated heptapeptide sequences denoted *a-b-c-d-e-f-g*. The positions *a* and *d* are occupied by hydrophobic amino acid residues (Val, Leu and Ile, Leu), while the *e* and *g* positions contain charged residues (Glu, Lys). The oppositely charged residues are responsible for the preferred formation of the heterodimers instead of homodimers. Concurrently, the selection of the charged residues may be used to control the mutual orientation of the peptide chains in the heterodimer.

While the E_1/K_1 heterodimer contains Glu in the *e* and *g* positions in all heptad repeats of the E_1 peptide and Lys residues in all the corresponding *e′* and *g′* positions of K_1 peptide, the ES_1/KS_1 heterodimer contains one Lys residue (*g*) in the third heptad of ES_1 and one Glu residue (*g′*) in the second heptad of KS_1. The oppositely charged residues in the corresponding heptads favor the antiparallel orientation of the peptide chains.

All peptides were prepared using a convergent approach: fragment condensation of the corresponding protected heptapeptide fragments. Nonlabeled peptides E_1, K_1, ES_1, KS_1 (Table 1) were prepared as described previously.[13]

Peptides E_2, K_2, ES_2, and KS_2 (Table 2) containing FRET labels at the *N*-termini of their peptide chains were prepared similarly using Fmoc-Glu(Edans) or Fmoc-Lys(Dabcyl) in the

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last condensation step. Analogously, peptides K_3 and KS_3 with Dabcyl labels at the C-terminus were prepared by starting the synthesis with Fmoc-Lys(Dabcyl). During the synthesis of the serine-containing peptides (ES_1, KS_1, ES_2, KS_2, KS_3), we used the corresponding pseudoproline dipeptides Fmoc-Lys(Boc)-Ser($\psi^{Me,Me}$Pro)−OH and Fmoc-Glu(OtBu)-Ser($\psi^{Me,Me}$Pro)−OH to increase the solubility of the protected heptapeptide fragments during the fragment condensation and the synthetic yield.[22,23]

In addition to the peptides described above, we also prepared diblock copolymers consisting of a peptide and a polymer block (Table 3). A semitelechelic poly(HPMA) polymer was synthesized using RAFT polymerization, and the terminal DTB group was substituted with a DBCO group, enabling subsequent metal-free click reactions with peptide azides (Scheme 1), as described previously.[21]

Table 3. Characteristics of the Polymer Conjugates

polymer conjugate	structure	M_w[a]	M_w/M_n[a]
PE_1	poly(HPMA)-DBCO-peptide E_1	60 000	1.20
PK_1	poly(HPMA)-DBCO-peptide K_1	49 300	1.25
PE_1+PK_1	coiled coil heterodimer	185 000	2.07
PES_1	poly(HPMA)-DBCO-peptide ES_1	46 000	1.39
PKS_1	poly(HPMA)-DBCO-peptide KS_1	43 500	1.25
PES_1+PKS_1	coiled coil heterodimer	81 000	1.90

[a]Molecular weights were determined by SEC using RI and MALS detection.

Circular Dichroism Spectroscopy. The secondary structures of the peptides and their equimolar mixtures were determined using CD.[24] It has been reported previously[13] that the individual E_1 and K_1 peptides adopt a random coil conformation in aqueous solutions with very low helicity. According to the molar ellipticity at 222 nm (Figure 3a), the equimolar mixture E_1/K_1 contained approximately 70% α-helix, indicating that the coiled coil heterodimer had formed. The stability of the heterodimer was characterized by the melting temperature of the α-helix (T_m = 61 °C) (Figure 3b). Specifically, peptides E_1 and K_1 changed their conformations from random coils to α-helices only after association with a coiled coil heterodimer.

A different situation was apparent for peptides ES_1 and KS_1. The CD spectra of the individual peptides ES_1 and KS_1 (Figure 4a) exhibited pronounced minima at 207 and 222 nm, which are typical for peptides with relatively high contents of α-helical secondary structures (60% and 51%, respectively). The helicity increased to 67% after mixing equimolar amounts of the peptides.

More obvious evidence of a strong interaction between the peptides in the solution was provided by measuring the thermal melting profiles of the peptides and their equimolar mixture. While the melting temperatures for peptides ES_1 and KS_1 were 34 and 58 °C, respectively, the equimolar mixture did not exhibit a characteristic sigmoidal melting curve between 5 and 95 °C (Figure 4b); consequently, no melting temperature was assigned. The significant increase of the melting temperature could likely be attributed to the formation of a relatively stable coiled coil heterodimer ES_1/KS_1.

In contrast to the previous peptide pair, ES_1 and KS_1 adopted α-helical secondary structures that retained the stability of their individual peptides. However, after mixing the two α-helices, they formed a more stable coiled coil heterodimer. This phenomenon was further explored using other physicochemical methods (SEC, ITC, FRET, AUC; see below).

Diblock poly(HPMA) copolymers formed by poly(HPMA) blocks connected with coiled coil linkers (PE_1/PK_1 and PES_1/PKS_1) exhibited similar behavior patterns to the corresponding peptides alone. While the CD spectra of the individual PE_1 and PK_1 copolymers revealed only very low contents of α-helical structures (20% and 28%, respectively), the peptides in their equimolar mixture contained 71% α-helix (Figure 5a). Analogous to the peptides ES_1 and KS_1, the respective peptides in the PES_1 and PKS_1 copolymers contained 44% and 30% α-helix, and their mixture contained 60% (Figure 5b), indicating the formation of a heterodimer (or, eventually, a coiled coil bundle with a higher association number). The thermal stability of the diblock poly(HPMA) copolymers PE_1/PK_1 and PES_1/PKS_1 and their equimolar mixture could not be estimated due to their very low ratio ($\Delta A/A$) caused by the absorption of the copolymer containing unstructured additive amide bonds and the DBCO group, which impeded the CD measurement.

Size-Exclusion Chromatography. The association behavior of the peptides in aqueous solution (PBS) was studied by SEC. Unfortunately, the data from the light scattering detector were inconclusive due to the low molecular weights and concentrations of the samples; therefore, it was not possible to determine the molecular weights of the peptides and their associates directly. Nevertheless, it was evident from the refractive index and UV data that there was a significant shift of the peaks to shorter elution times after mixing of both the E_1/K_1 (Figure 6a) and ES_1/KS_1 (Figure 6b) peptides. Therefore,

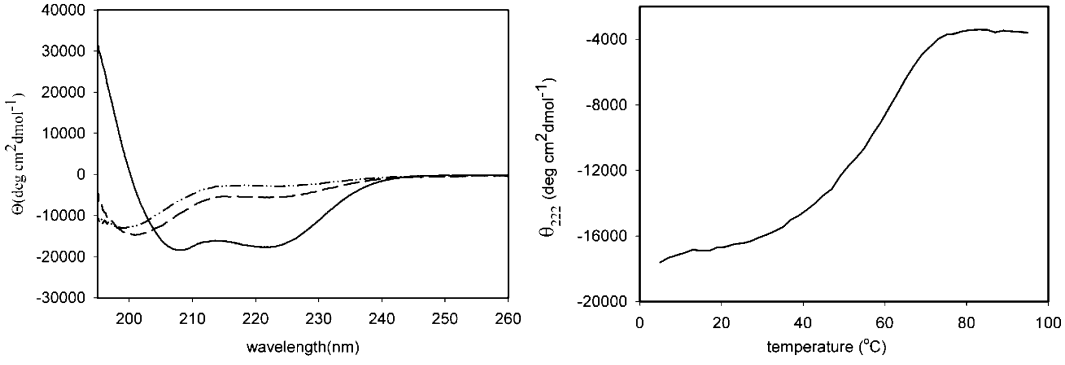

Figure 3. (a) CD spectra of peptides E_1 (dot-dash), K_1 (dash) and their equimolar mixtures (solid line). (b) Thermal melting profile of equimolar mixture of peptides E_1/K_1.

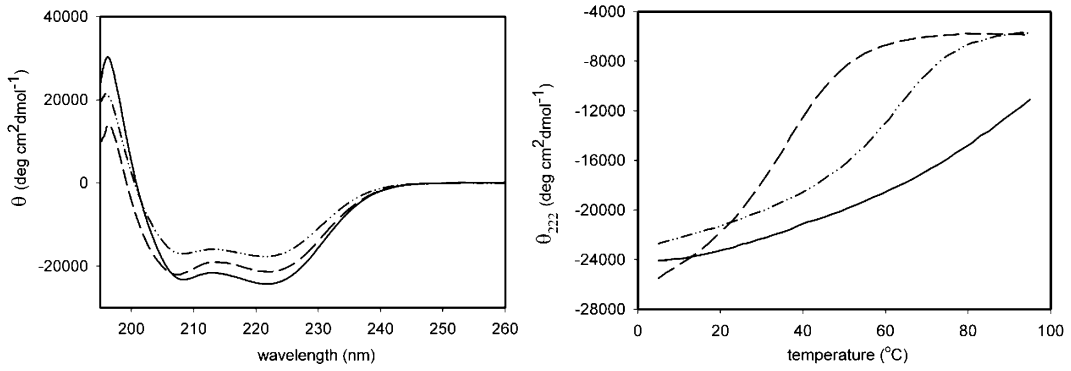

Figure 4. (a) CD spectra of peptides ES_1 (dash), KS_1 (dot-dash) and their equimolar mixture (solid line). (b) Thermal melting profiles of the peptides and their equimolar mixture.

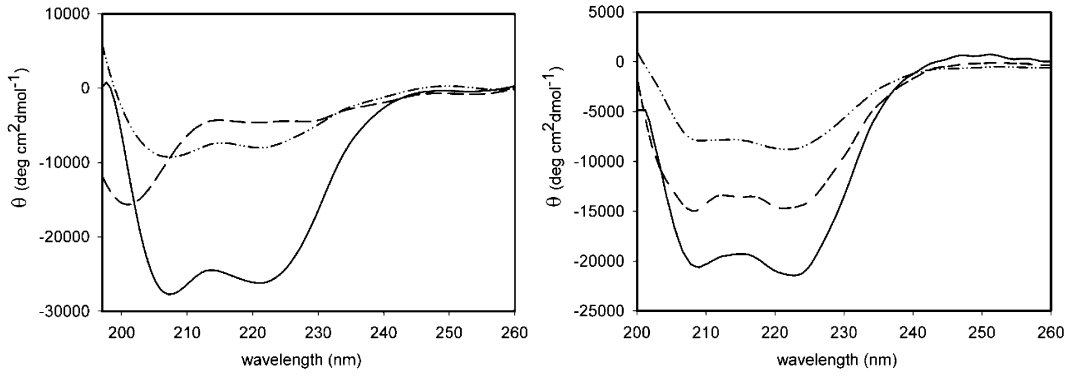

Figure 5. CD spectra of polymer−peptide conjugates and their equimolar mixtures. (a) PE_1 (dash), PK_1 (dot-dash) and their equimolar mixture (solid line). (b) PES_1 (dash), PKS_1 (dot-dash) and their equimolar mixture (solid line).

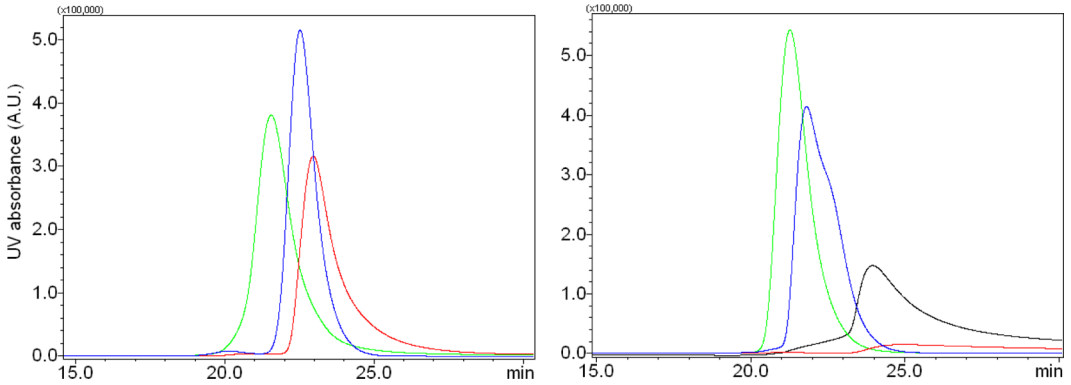

Figure 6. SEC chromatograms of the peptides (a) E_1 (blue), K_1 (red) and their equimolar mixture (green) and (b) ES_1 (blue), KS_1 (red), KS_1 at pH 4.4 (black) and their equimolar mixture (green) on Superdex Peptide column in PBS (pH 7.4) monitored by UV detector at 220 nm. The concentration of the peptides was 0.4 mg mL^{-1} and the injection volume was 50 μL.

the increasing hydrodynamic diameter may be attributed to the formation of the coiled coil heterodimers. Interestingly, the SEC profile of peptide KS_1 indicates the strong adsorption of the analyte to the stationary phase of the column due to the number of nonprotonated amino groups at pH 7.4. After acidifying the mobile phase to pH 4.4, this sorption was substantially eliminated, and the peak corresponding to the peptide KS_1 had a standard shape (Figure 6b, black curve).

Similar to the peptides, the chromatograms of the corresponding diblock copolymers also exhibited significant changes upon mixing of the individual copolymers PE_1/PK_1 (Figure 7a) and PES_1/PKS_1 (Figure 7b). In contrast to the much smaller peptides, it was already possible to measure the absolute values of the molecular weights based on the data from the light scattering detector for the copolymers and their mixtures (Table 3). Although it is difficult to directly assess the association number of the complexes formed in the copolymer mixtures, it is evident that there was a significant increase in molecular weight after mixing the individual poly(HPMA)-peptide copolymers in both studied systems, documenting the self-assembly and conjugation of the two poly(HPMA) chains. Broadening of the peaks (polydispersity index about 2) indicates that probably not all polymer chains were self-assembled due to the steric hindrance between the polymer coils; at the same time, higher oligomers (trimers or tetramers) are also formed as confirmed by sedimentation analysis and

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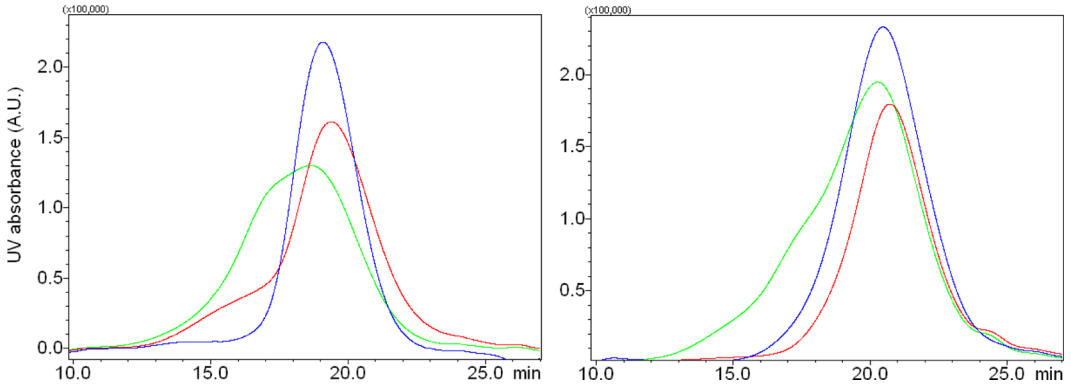

Figure 7. SEC chromatograms of the copolymers (a) PE_1 (blue), PK_1 (red) and their equimolar mixture (green) and (b) PES_1 (blue), PKS_1 (red) and their equimolar mixture (green) on MicroSuperose 6 column in PBS monitored by UV detector at 220 nm. The concentration of the copolymers was 1.34 mg mL^{-1}, and the injection volume was 50 μL.

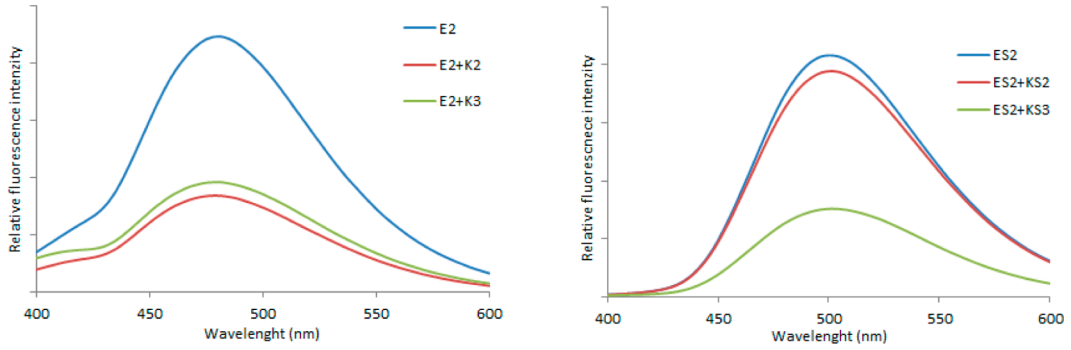

Figure 8. Fluorescence emission spectra of peptides (a) E_2, E_2+K_2, E_2+K_3 and (b) ES_2, ES_2+KS_2, ES_2+KS_3 were measured in PBS at 0.1 mg mL^{-1} concentration using an excitation wavelength of 335 nm.

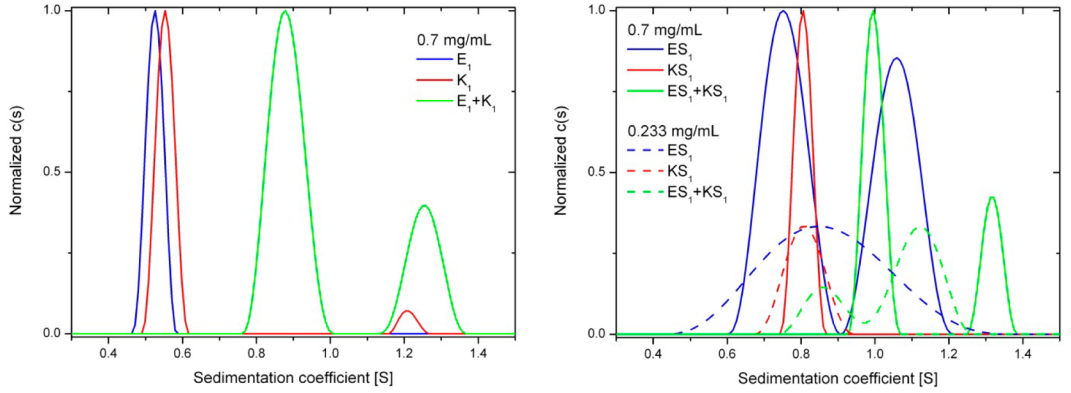

Figure 9. Sedimentation analysis of peptides (a) E_1 (blue), K_1 (red) and their equimolar mixture (green) and (b) ES_1 (blue), KS_1 (red) and their equimolar mixture (green) at 20 °C in PBS at 0.7 mg mL^{-1} (solid line) and 0.233 mg mL^{-1} (diluted three times, dashed line) total loading concentration; continuous size distributions c(s) were normalized and in (b) adjusted relative to different loading concentrations.

ITC. Considering this and the fact that the association number depends both on the temperature and concentration, exact calculation of the association number only from SEC results would not be serious.

Orientation of the Peptide Chains in the Coiled Coil–FRET Experiments. Fluorescence (or Förster) resonance energy transfer (FRET) is a phenomenon enabling an energy transfer between two chromophores.[25] A donor chromophore, which is initially in its electronic excited state, may transfer energy to an acceptor chromophore through nonradiative dipole–dipole coupling. The efficiency of this energy transfer is inversely proportional to the sixth power of the distance between the donor and acceptor, making FRET extremely sensitive over small distances. Consequently, FRET efficiency can be used to determine whether two fluorophores are within a certain distance of each other.

As previously mentioned (design and synthesis of the peptides), the irregularity in the structure of the heptads at the g and g' positions in the ES_1/KS_1 coiled coil dimer (Figure 2b) (i.e., Lys replaced by Glu and vice versa) should theoretically lead to an antiparallel orientation of the peptide chains. To verify this hypothesis, 5-[(2-aminoethyl)amino]-naphthalene-1-sulfonic acid (Edans) was covalently bound to the *N*-termini of peptides E_2 and ES_2 as a donor. 4-[4-

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(Dimethylamino)phenylazo]-benzoic acid (Dabcyl) was bound to the *N*-termini of peptides K_2 and KS_2 and to the *C*-termini of K_3 and KS_3 as an acceptor. If the labeled ends of the peptides are close enough, FRET should occur between the donor and the acceptor, resulting in a substantial decrease in the fluorescence intensity of the first.

The decrease in fluorescence intensity was approximately the same after mixing the aqueous solution of peptide E_2 (Edans at the *N*-terminus) with K_2 (Dabcyl at the *N*-terminus) and with K_3 (Dabcyl at the *C*-terminus) (Figure 8a). However, after adding peptide KS_2 (Dabcyl at the *N*-terminus) to peptide ES_2 (Edans at the *N*-terminus), almost no change in the fluorescence intensity was observed compared to the significant decrease caused by adding the equimolar amount of peptide KS_3 (Dabcyl at the *C*-terminus) to ES_2 (Figure 8b). Therefore, while the orientation of the peptides in the E_1/K_1 coiled coil heterodimer is mostly random, the preferred orientation in the ES_1/KS_1 heterodimer is antiparallel.

Analytical Ultracentrifugation. To analyze the associative behavior of the peptides in aqueous solution in more detail, sedimentation analyses of the individual peptides and their equimolar mixtures were performed in PBS buffer using an analytical ultracentrifuge.[26−28] In a sedimentation velocity experiment at 0.7 mg mL^{-1}, both E_1 and K_1 sedimented as unimers with $s_{20,w}$ sedimentation coefficient values of 0.53 and 0.56 S, respectively (Figure 9a). However, a small fraction of peptide K_1 molecules formed oligomers that could be best described as homotrimers or homotetramers, based on their sedimentation coefficient values and depending on the chosen shape of the sedimenting particle. The E_1/K_1 mixture sedimented at a distinctively higher $s_{20,w}$ value of 0.90 S, corresponding to the anticipated coiled coil dimer; however, a significant number of molecules formed trimeric or tetrameric complexes. These observations were corroborated using sedimentation equilibrium analysis, yielding weight-average molecular masses of 3.5 kDa for E_1, 3.4 and 6.5 kDa for K_1, and 7.5 and 10 kDa for E_1/K_1 mixture; within the precision of this method, these values correspond well with the masses of unimers, dimers, or trimers of the given peptides (data not shown). Obviously, peptides E_1/K_1 form coiled coil dimers, and peptide K_1 is capable of forming higher homo-oligomers that might contribute to the formation of higher hetero-oligomers within the E_1/K_1 mixture.

Different results were obtained for ES_1 and KS_1. Both peptides tend to form homo-oligomers at 0.7 mg mL^{-1}. Peptide KS_1 sediments as a single particle with an $s_{20,w}$ value of 0.81 S, clearly corresponding to its homodimer, and peptide ES_1 sediments as two distinct particles with $s_{20,w}$ values of 0.77 and 1.07 S, indicating homodimer and homotrimer formation (Figure 9b). The equilibrium between these two ES_1 forms is reflected in the broad size distribution observed for an ES_1 sample diluted three times (Figure 9b, blue dashed line); its peak is shifted to a value of 0.88 S, corresponding to an average value of the dimer/trimer species. For the equimolar mixture of the ES_1/KS_1 peptides, the size distribution of the sedimenting species is shifted toward higher $s_{20,w}$ values of 1.02 and 1.34 S, most likely corresponding to heterotrimers and heterotetramers (Figure 9b, full green line). After dilution, the equilibrium between the trimeric and tetrameric species is visible, in addition to the dissociation of trimeric species to dimers (Figure 9b, dashed green line). The sedimentation equilibrium analysis of the diluted samples confirms that these observations have weight-averaged molecular masses of 7 kDa for ES_1, 7.4 kDa for KS_1, and 10 kDa for the ES_1/KS_1 mixture, corresponding to the predominant dimeric (ES_1, KS_1) or trimeric (ES_1/KS_1) peptides (data not shown).

Isothermal Titration Calorimetry. ITC experiments were performed for the E_1/K_1 and ES_1/KS_1 peptides.[29−31] A set of experiments at different temperatures was carried out to obtain the complete thermodynamic signature of the coiled coil complex formation. For both types of systems (ES_1/EK_1 and E_1/K_1 and those not presented on the graph), exothermic titration peaks were observed in the thermograms (Figure 10). After integrating, the exothermic process was visible on the curve at molar ratios between 0.4−2.0 (Figure 11a,b). We attributed this process to the complexation of the peptides.

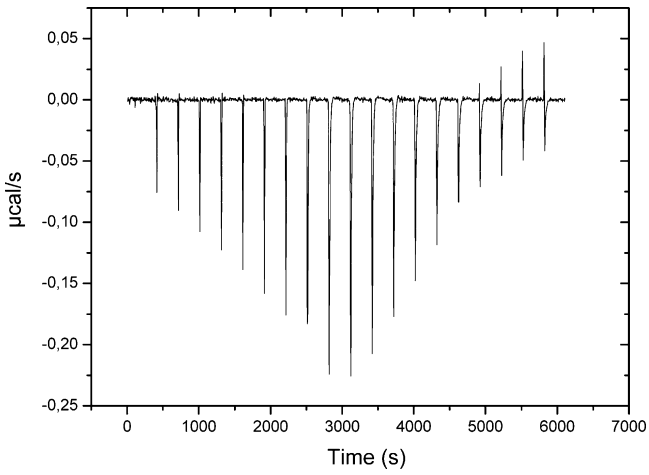

Figure 10. ITC thermogram-heat signal as a function of time for the ES_1/KS_1 system at T = 25 °C.

The thermodynamics of protein−protein interactions can depend on the ambient temperature, and the known variation in the thermodynamics relative to the temperature can provide useful information regarding the mechanism of complex formation.

Numerous features could be detected after analyzing the ITC data obtained at different temperatures. From the "two sets of sites" model fitting (Figure 11a,b), the value of the stoichiometry parameter n_2 has been obtained. For the E_1/K_1 peptides, this number evolves from n_2 = 0.50 at 15 °C to 0.92 at 37 °C. This behavior can be explained by the compositional heterogeneity of the complexes; we assume that the heterodimers, in addition to a portion of the heterotrimers and heterotetramers, are present in the solution, agreeing with the conclusions reported above by the AUC method at 20 °C. For the ES_1/KS_1 peptides, n_2 varies from 0.6 to 0.7 up to 50 °C, similar to the AUC results, showing that heterotrimers and heterotetramers are only observed for an equimolar mixture of ES_1/KS_1 peptides.

The increasing temperature shifts the stoichiometry of the binding process due to the reduced polydispersity of the system. Another feature of the curves is worth mentioning. The binding isotherm at low temperatures displays a sharp and pronounced transition at small molar ratios (0−0.5), followed by the main transition process characterizing the formation of the peptide heterocomplexes (Figure 11a,b). The curvature and amplitude of the first transition decreases with increasing temperature. To understand the origin of this process, the procedure for the isothermal titration should be recalled. In the

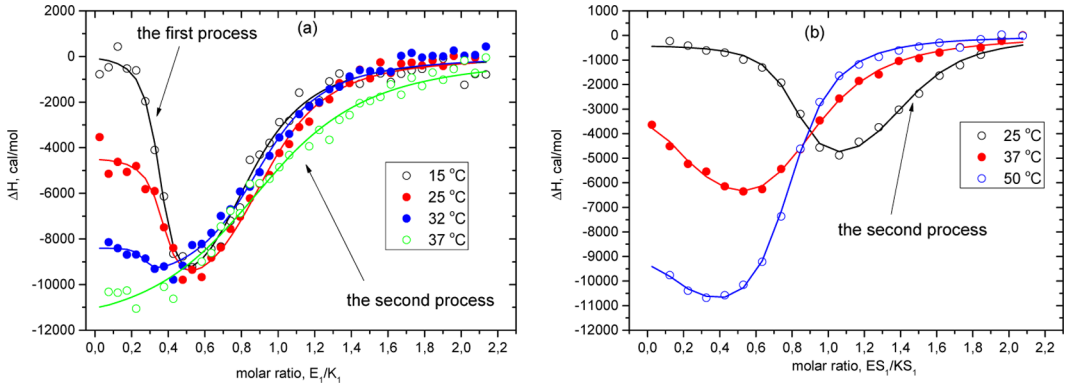

Figure 11. Measured (symbols) and fitted (solid lines) ITC data for the E_1/K_1 system (a) and the ES_1/KS_1 system (b).

beginning of the titration, a pure solution of peptide KS_1 or K_1 was placed in the calorimeter cell at a certain temperature. Based on the analytical ultracentrifugation data at 20 °C, the K_1 protein exists as a unimer with a small fraction of homotrimers or homotetramers, while KS_1 exists as a homodimer in the solution. The consecutive addition of a small amount of the peptide ES_1 or E_1 should affect the state of KS_1 (K_1) in two different ways. From one side, the interaction between ES_1 and KS_1 should lead to the formation of heterocomplexes (dimers, trimers, etc.), while from another side, it must reduce the number of monomolecular peptide in the system, continuously shifting the equilibrium of the dimerization process for second peptide toward dissociation. The combination of these two complementary processes might be observed on a titration curve (Figure 11a,b). Therefore, one can assume that the dissociation of homodimers dominates during the first exothermic process, while the second process is defined by the formation of heterodimers for E_1/K_1 system or heterotrimers/heterotetramers for the ES_1/KS_1 system. Therefore, the temperature sensitivity of the first transition process and the shift in the stoichiometry of the second one implies that the KS_1 and K_1 peptides have different initial states at different temperatures. The size distribution of the peptides becomes uniform and unimodal as the temperature increases.

CONCLUSIONS

The associative behavior of two pairs of peptides and their conjugates with poly(HPMA) in aqueous solutions was studied using SEC, CD spectroscopy, ITC, FRET, and AUC. According to the CD measurements, peptides E_1 and K_1 and their polymer conjugates PE_1 and PK_1 had a random coil conformation in aqueous solutions, while the equimolar mixtures of both the free peptides and the peptides in the polymer−peptide conjugates adopted α-helical structures, indicating the formation of coiled coil peptide heterodimers. In contrast, peptides ES_1 and KS_1 and the peptides in their polymer conjugates (PES_1, PKS_1) exhibited a high α-helix content as individual peptides (or polymer−peptide conjugates); preparing the equimolar mixtures increased the α-helix content further while increasing the melting points of the corresponding helices. The FRET experiments with the corresponding fluorescently labeled peptides revealed that while the orientation of the peptides in the E_1/K_1 coiled coil heterodimer is mostly random, the antiparallel orientation predominates in the ES_1/KS_1 hetero-oligomer. The ITC and AUC studies showed that the associative behavior of both peptide pairs is more complex than can be described by a simple equilibrium between the peptide unimers and heterodimers. In addition to the anticipated heterodimers, higher oligomerization degrees (trimers and tetramers) were present in the equimolar mixtures of the peptides. The fraction of the higher hetero-oligomers was elevated in the ES_1/KS_1 mixture compared to the E_1/K_1 system. Furthermore, the oligomerization state of the peptides was strongly affected by both concentration and temperature.

The results of this work can be used to improve the design of new generations of macromolecular therapeutics and diagnostics specifically targeted using recombinant protein ligands containing coiled coil heterodimers (or hetero-oligomers) as linkers between the polymer carrier and the targeting protein. The major advantage of these noncovalent linkages between proteins and synthetic polymer drug carriers compared to the covalent modification of biomacromolecules lies in their simple, universal and nondestructive preparation, as well as in the well-defined structure of the resulting macromolecular system.

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Notes

The authors declare no competing financial interest.

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