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ARTICLE

Polypseudopeptides with Variable Stereochemistry: Synthesis via Click-Chemistry, Postfunctionalization, and Conformational Behavior in Solution

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ABSTRACT: Polypseudopeptides with well-defined stereochemistries have been synthesized from readily available amino-acid-based building blocks by connecting (L,L)- or (L,D)-dipeptide AB-monomers carrying azide and alkyne termini via triazole amide-isosteres efficiently formed in the course of the "click" reaction. Deprotection of the thus-prepared lysine-based polypseudopeptides of both *all*-(L)- and (D)-*alt*-(L)-stereochemistries afforded water-soluble polymers with ionizable amino side chains, which could be fully labeled with pyrene chromophores via quantitative amide bond formation. The conformational behavior of the deprotected as well as the pyrene-labeled polymers was investigated using UV/vis, CD, and fluorescence spectroscopies. On one hand, the free polyamines display pH-dependent conformations in water. On the other hand, the pyrene-labeled polypseudopeptides change their conformation in response to varying organic solvent composition. Whereas the strictly alternating polypseudopeptides structurally resemble channel-forming peptides, such as the Gramicidin family, the incorporation of (D)-configured amino acids as well as triazole amide-isosteres should lead to interesting new materials for bioapplications.

Introduction

In natural as well as artificial macromolecules, structure–property relationships are manifested by a hierarchical construction plan starting from the monomer unit, its integration into larger covalent strands with preferred chain conformations, and their noncovalent intra- and intermolecular superstructures.[1] This bottom-up structural evolution is perhaps best illustrated when looking at the primary, secondary, tertiary/quaternary structure of peptides.[2] For decades, in particular, medicinal chemists have been engaged in modifying and mimicking peptides[3] to enhance their pharmacological properties such as biodegradability, bioavailability, and suppressed immune response. Recently, the successful incorporation of triazole amide-isosteres into a peptide,[4] as demonstrated by Ghadiri,[5] and the synthesis of nonpeptidic foldamers, as shown by Arora,[6] point out the synthetic feasibility and structural versatility of these pseudopeptides and demonstrated their incorporation into polypeptide backbones. Such triazole-based peptide mimics are heavily pursued targets for various biological and pharmaceutical applications.[4]

Another important structural feature in peptides is the stereochemistry at the α-carbon. This is nicely illustrated when comparing *all*-(L)-peptides, which commonly adopt the α-helical secondary structures used in proteins to arrange functional moieties at the helix exterior, with alternating (D)-*alt*-(L)-peptides able to form hollow *β*-helices with cation-transporting ability, for example, Gramicidin channels.[7] Obviously, the introduction of D-configured amino acids largely alters the secondary structure formation and in addition leads to vastly different pharmacological characteristics. Although the synthesis of polypeptides with high molecular weights and narrow polydispersities has been developed on the basis of the living ring-opening polymerization of α-amino acid *N*-carboxy anhydrides (NCAs),[8] this method cannot be adopted to prepare strictly alternating (D)-*alt*-(L)-polypeptides. One way to address this problem is to connect alternating (D,L)-dipeptide (or higher, even-numbered oligopeptide) monomers in a step-growth polymerization process. However, such fragment condensation requires reaction conditions that proceed without the loss of the stereochemical information, that is, epimerization. For this reason, we chose to utilize the Cu-catalyzed 1,3-dipolar cycloaddition of terminal alkynes and azides, the so-called "click" reaction[9−11] to connect (D,L)-dipeptide monomers via triazole amide-isosteres.[12,13] Here we report on the synthesis and postfunctionalization of these novel (D)-*alt*-(L)-polypseudopeptides composed of alternating (D,L)-dipeptide units connected by triazole moieties. The prepared polymers were furthermore characterized with regard to their solution conformation.

Results and Discussion

Design Considerations. The targeted triazole-containing polypseudopeptides are composed of short, stereochemically defined peptide segments connected via triazole moieties. In principle, such polymers can be obtained via either copolymerization of A_2- and B_2-monomers or polymerization of an AB-monomer. However, only the AB-monomer strategy enables transfer of the peptide segments' stereochemical information into one and the same direction of the pseudopeptide backbone (Figure 1). In addition, AB-polycondensation offers the advantage of an inherently balanced stoichiometry,[14] assuring for high degrees of polymerization, even on a small synthetic scale.

The generation of a hydrophilic, potentially pH-responsive polymer with a Gramicidin-type (D,L)-alternating stereochemistry requires the use a dipeptide building block with basic or acidic side chain functionalities. The use of properly protected lysine-based dipeptide monomers (Figure 1) meets all of these requirements and moreover allows for facile side-chain postfunctionalization of the polymer. Note that in

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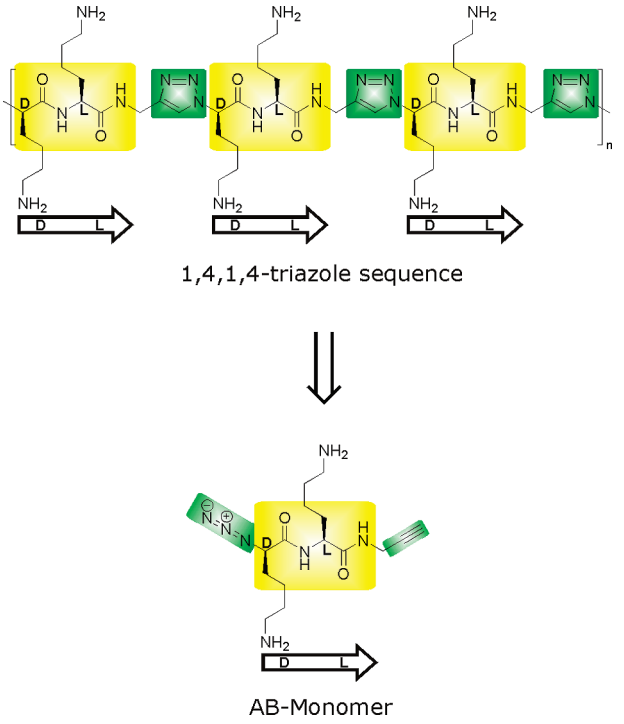
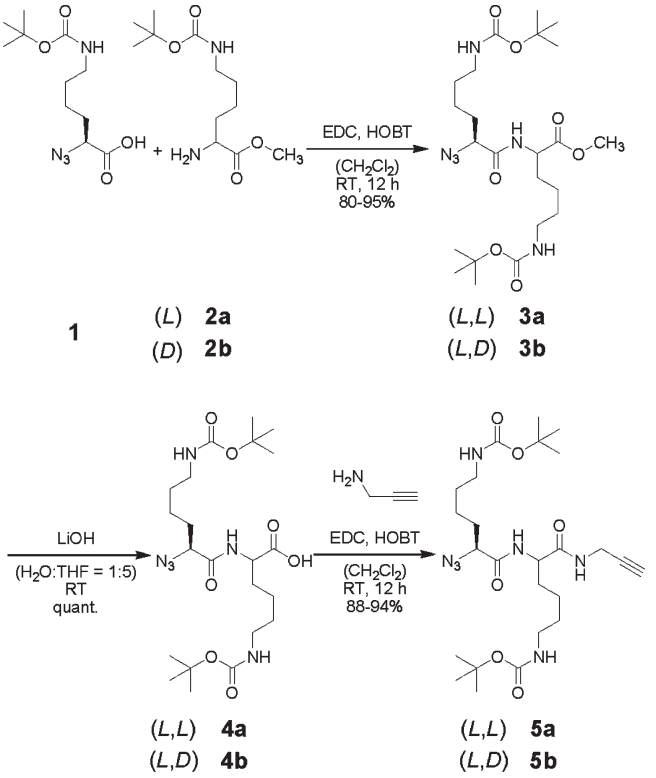


Figure 1. Design of an appropriate AB-monomer suitable for the incorporation of (D)-*alt*-(L)-stereochemistry and into the same main chain direction of the polymer backbone.

principle our approach could also be applied to elongated, yet even-numbered oligopeptides, for example, to encode specific sequence information into the resulting pseudopeptide backbone. Introduction of the A- and B-functionalities, that is, azides and terminal acetylenes, into the dipeptide monomer segments has to be accomplished without loss of stereochemical information, that is, epimerization. The azide functionality can be introduced by using an α-azido acid, readily available from its corresponding amino acid via Cu-catalyzed azide group transfer at the *N*-terminus under retention of the absolute configuration (Supporting Information), whereas the acetylene can conveniently be incorporated by amide coupling propargylamine to the C terminus. Subsequent polymerization of the AB-"click"-monomer should give access to the desired *all*-(L)- as well as (D)-*alt*-(L)-polypseudopeptides.

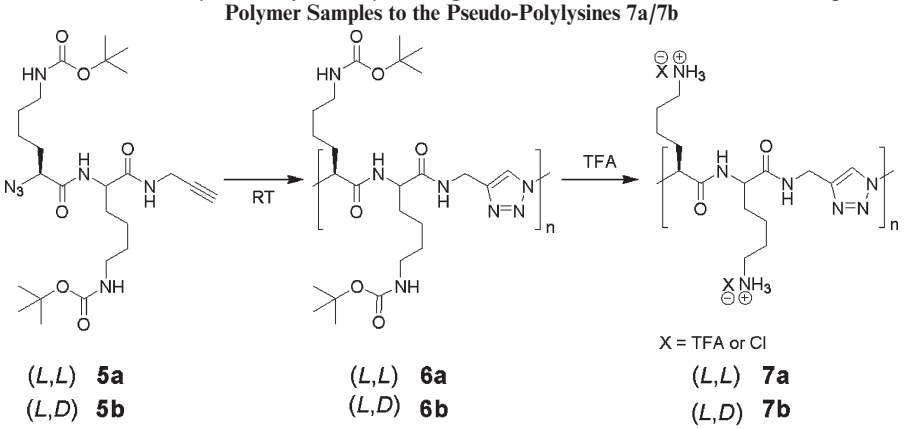
Monomer Synthesis. In our initial approach to the synthesis of the desired monomer, 2-chlorobenzyloxycarbonyl (2Cl-Z) was chosen to be the *Nε*-protecting group. Whereas the robustness of this protecting group was appealing in view of the compatibility with the azide transfer, it turned out to be less suitable because its limited solubilizing ability caused precipitation in the early stages of the subsequent polymerization reaction (Supporting Information). To overcome this problem and increase the solubility of the polymer, the 2Cl-Z group was replaced by the *tert*-butyloxycarbonyl (Boc-) group, requiring alteration of the monomer synthesis. The coupling of azido-(L)-Lys(Boc) (**1**) with (L)- and (D)-Lys-(Boc)-Me (**2a**/**2b**) (Supporting Information) proceeded smoothly and gave the resulting dipeptides **3a/3b** in good-to-excellent yields after purification via column chromatography (Scheme 1). Subsequent saponification of the methyl ester gave the free acids **4a**/**4b** in quantitative yields. The acids were then coupled to propargylamine to give the desired AB-"click"-monomers azido-(L)-Lys(Boc)-(L)-Lys-(Boc)-propargylamide (**5a**) and azido-(L)-Lys(Boc)-(D)-Lys-(Boc)-propargylamide (**5b**) in good-to-excellent yields.

Scheme 1. Synthesis of Boc-Protected *all*-(L)- and (D)-*alt*-(L)-AB-"Click"-Monomers 5a/5b, Respectively, Starting from (L)-Azido-Lys-(Boc) 1 and (L)- or (D)-Lys(Boc)-Me 2a/2b



"Click"-Polyaddition. The purified monomers **5a/5b** were subjected to various polymerization runs (Scheme 2). The polymerizations were carried out in DMF or DMF−water mixtures (up to 50 vol % H_2O) and monomer concentration ranges from 0.44 to 1.4 mol/L at room temperature over periods from 3 to 14 days, resulting in polymers with broad polydispersities ($1.5 < PDI < 7.8$) and molecular weights varying from $19\,000 < M_w < 180\,000$ g/mol (as determined by GPC analysis in DMF at 70 °C, calibrated with polystyrene standards, RI-detection; see Table S2 in the Supporting Information). In all reactions, the monomer was dissolved in the minimum amount of DMF necessary to obtain a solution, which could be magnetically stirred. When the copper catalyst was derived from $CuSO_4$, highly concentrated aqueous solutions of sodium ascorbate and $CuSO_4$ were added (standard conditions). When tetrakis-(acetonitrile)copper(I) hexafluorophosphate was used as copper catalyst, the addition of water was not necessary (water-free conditions). In all reactions, copper wire was added and *N*,*N*′-dimethylethylenediamine was used as ligand. As expected, the use of the Boc-protecting group avoided precipitation during the polymerization, even in the presence of water. Instead, the reaction mixtures started to become highly viscous after ∼6 h. Monitoring of the reaction via GPC revealed that once the reaction mixture had become highly viscous, longer stirring (even under dilution of the sample) led to no significant further polymerization. The general workup procedure consisted of a repetitive precipitation in aqueous EDTA solution, followed by filtration. The crude product was swollen and dialyzed in MeOH using a dialysis tube with a molecular weight cutoff (MWCO) of 25 000 g/mol. In most cases, dialysis yielded pure high-molecular-weight material, which was analyzed by GPC (in DMF at 70 °C, calibrated with polystyrene standards, RI-detection). GPC traces of representative polymer

Scheme 2. Polymerization of Monomers 5a/5b to Polymers 6a/6b Using Different "Click" Conditions and Subsequent Boc-Deprotection of the Polymer Samples to the Pseudo-Polylysines 7a/7b



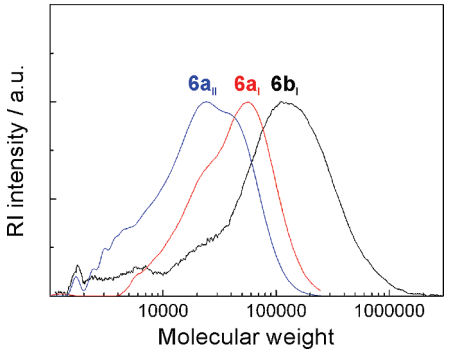


Figure 2. GPC traces of polypseudopeptides **6a$_{I}$**, **6a$_{II}$**, and **6b$_{I}$** (all values are normalized) showing the polymers' apparent molecular weight distributions (GPC in DMF, 0.5 wt % LiBr, at 70 °C, calibrated with polystyrene standards). Roman subscripts refer to different polymerization batches. (See Table S2 in the Supporting Information.)

samples (Figure 2) show typical broad peaks, in line with a classical step-growth polymerization mechanism.[14] In view of the rather large degrees of polymerization, the nature of the end groups could not be discerned, for example, by ^{1}H NMR because of negligible signals.

No influence of the monomer stereochemistry on the degree of polymerization of the resulting polymer was observed because both (L,L)-monomer **5a** as well as (L,D)-monomer **5b** yielded polymers of comparable length. Even reactions run under water-free conditions did not significantly improve the molecular weight of the polymers. As indicated by the different monomer and hence polymer solubilities, polymerization of the Boc-protected monomers gave rise to much higher molecular weights, as compared with polymerizing the 2Cl-Z-protected monomer.

Model Compounds. The analysis and structural investigation of a new polymer family oftentimes suffers from the drawback that signals observed using various characterization techniques, that is, NMR, CD, cannot be assigned to particular atoms or structural motifs. To facilitate this assignment and hence structural analysis, comparison of the polymer signals with those of an appropriately designed model compound can be very helpful. In our case, the monomers were not sufficient models of the polymer because the triazole unit (one major component of the polymer backbone being formed within the polymerization) is not present. Therefore, model compounds **8a** and **8b** featuring a central triazole unit flanked by two lysine residues were synthesized (Supporting Information), and the combination with the Boc-deprotected monomers **9a** and **9b** represents a

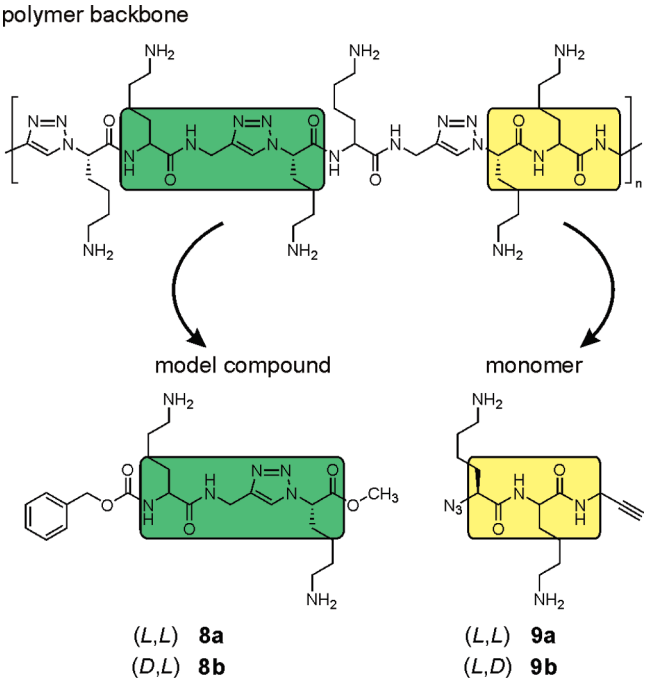


Figure 3. Key structural elements of the polymer backbone (highlighted in green and yellow) mimicked by Boc-deprotected *all*-(L) and (D)-*alt*-(L) model compounds **8a**/**8b** and monomers **9a**/**9b**.

suitable model system, mimicking the key structural features of the polymer backbone (Figure 3).

Postfunctionalization. For further studies, the polymers **6a$_{I}$**, **6a$_{II}$**, and **6b$_{I}$** were Boc-deprotected (Scheme 2). The Boc-deprotection proceeded smoothly and yielded the polymers after dialysis in water using a dialysis tube with MWCO of 25 000 g/mol. A part of the polymer sample was also subjected to exchange the trifluoroacetate counterions, present from TFA treatment, to chloride counterions, by dissolving the polymer in 1 M HCl and subsequent dialysis.

Standard polymer characterization by GPC proved to be difficult because neither UV- nor RI-detection was feasible because of the lack of chromophores and the similar refractive indices of the polymer and the solvent, respectively. The rather broad molecular weight distribution prohibited analysis by MALDI-TOF mass spectrometry. However, complete deprotection was verified by ^{1}H NMR confirming the absence of the Boc-group and reflecting the remaining polymer backbone structure (Figure 4).

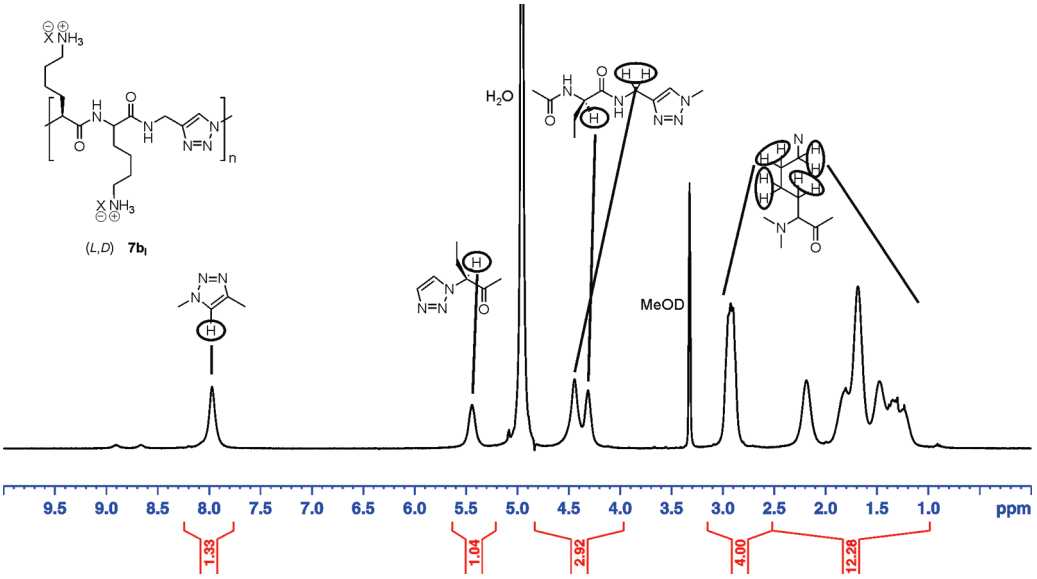


Figure 4. ^{1}H NMR spectrum of deprotected polypseudopeptide **7b$_I$** (MeOH-d_4, 25 °C).

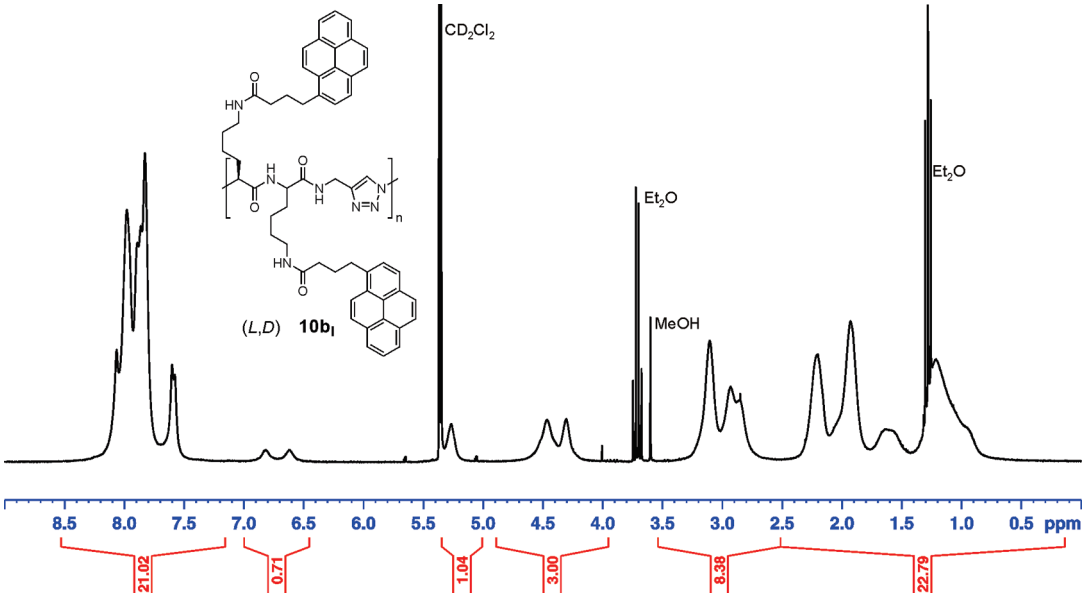
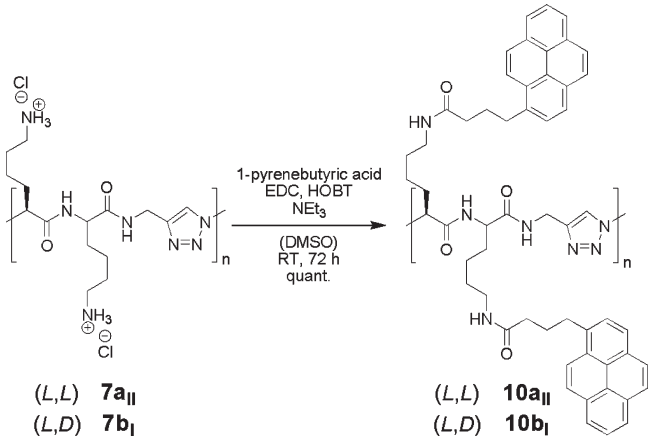


Figure 5. ^{1}H NMR spectrum of pyrene-labeled (D)-*alt*-(L)-polypseudopeptide **10b$_I$** in CD_2Cl_2: TFA-d_1 = 5:1 (25 °C).

Scheme 3. Post-Functionalization of Polymers 7a$_{II}$ and 7b$_I$ with 1-Pyrenebutyric Acid to Yield Side-Chain Labeled Polymers 10a$_{II}$ and 10b$_I$



After successful deprotection of the side chains and dialysis (Figure S1 in the Supporting Information), **7b$_I$** was reacted with excess 1-pyrenebutyric acid using EDC and HOBT as coupling reagents (Scheme 3). Whereas the use of triethylamine was necessary to deprotonate the amines, DMSO proved to be the solvent of choice because DMF could not efficiently dissolve the polymer, necessitating the addition of water.

After the reaction, the resulting mixture was directly dialyzed in CH_2Cl_2/MeOH 1:1 using a dialysis tube with MWCO of 25 000 g/mol. Dialysis gave the desired polymers as a fawn solid in quantitative yield. In comparison with the Boc-protected polymers **6a$_{II}$** and **6b$_I$**, the peaks of the pyrene-labeled polymers **10a$_{II}$** and **10b$_I$** in the GPC were slightly shifted to lower molecular weights (Figure S6 in the Supporting Information), indicating more compact structures when comparing their hydrodynamic volume. Analysis of the ^{1}H NMR spectrum of polypseudopeptide **10b$_I$** (Figure 5), comparing backbone and side-chain integration, indicates practically quantitative side chain labeling.

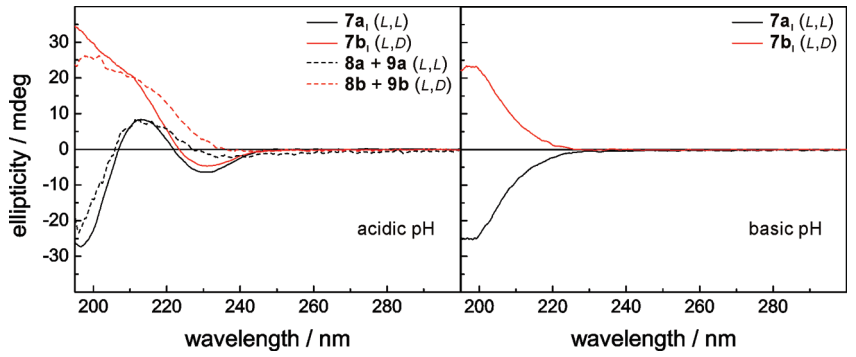


Figure 6. CD spectra of polymers **7a$_I$** and **7b$_I$** under acidic (pH = 2 to 4) and basic (pH = 11 to 12) conditions (solid lines) at 25 °C. Acidic polymer spectra are in comparison with model spectra obtained by 1:1-addition of the spectra of the respective model compounds **8a** or **8b** and the monomers **9a** or **9b** (dashed lines). Samples were measured at the following concentrations: **7a$_I$** (4.88×10^{-4} mol/L), **7b$_I$** (4.95×10^{-4} mol/L) (both as referred to monomeric repeat unit), **8a** (8.71×10^{-4} mol/L), **8b** (8.57×10^{-4} mol/L), **9a** (7.52×10^{-4} mol/L), and **9b** (7.27×10^{-3} mol/L). All spectra are normalized to a concentration of 1×10^{-3} mol/L.

Conformational Behavior in Solution. The conformational behavior of the Boc-deprotected polypseudopeptides **7a$_I$** and **7b$_I$** was monitored by CD spectroscopy. The polymers were measured at concentrations around 1×10^{-3} mol/L (as referred to monomeric repeat unit). Aggregation effects were excluded by dilution series at acidic and basic pH, showing linear Lambert−Beer-type behavior (Figures S2 and S3 in the Supporting Information). Variable temperature experiments at unmodified pH ($5 \leq$ pH ≤ 7) revealed small, reversible changes in the CD spectra within a temperature range of 5−45 °C. Temperatures above 45 °C led to more pronounced, yet irreversible, changes in the CD spectra (Figure S4 in the Supporting Information) originating from currently unidentified denaturation processes.

By analogy to the pH-dependent structures of polylysine,[16] polymers **7a$_I$** and **7b$_I$** are expected to adopt a random coil structure with a characteristic and known CD signature in an acidic environment because of the Coulomb-repulsion of the charged side chains. At first, it is not clear if the observed polymer CD spectra in our case are associated with a random coil structure because no comparison is possible for this currently unknown backbone type. Clearly, the optical transitions of the backbone are originating from both amide and triazole chromophores. These chromophores are contained in monomers **9a**/**9b** and model compounds **8a**/**8b**, and because they are too small to form any secondary structure, the addition of their CD spectra should provide a suitable model spectrum for the random coil structure of the polymer. Again, aggregation effects in these small molecules were ruled out by dilution series. (See the Supporting Information.) The simple mathematical addition of the CD spectra of monomer and model compound (Figure S5 in the Supporting Information), that is, **8a** + **9a** and **8b** + **9b**, in a 1:1 ratio and overlay with the corresponding polymer spectra are shown in Figure 6 (left). Indeed, the polymer spectra are very similar to the simulated composite spectra, pointing to a random coil structure of both polymers **7a$_I$** and **7b$_I$** in an acidic environment. The stereochemical influence of side chain substitution is clearly reflected in the opposite ellipticities measured in the far UV around 200 nm, illustrating the opposite configuration adjacent to the amide chromophores. In contrast, the spectra are rather similar at higher wavelengths around 230 nm, possibly associated with the triazole transitions. Note that the adjacent side chain (in α-position to N-1 of the triazole) is (L)-configured for the polymers as well as both model compounds **8a**/**8b**, which should not be affected by the different stereochemistries. Raising the pH to 11 to 12 irreversibly changes the polymers' CD spectra (Figure 6, right). The resulting spectra of **7a$_I$** and **7b$_I$** behave as mirror images, reflecting the opposite conformations. In a basic environment, the side chains are deprotonated, and the polymer may be able to adopt an ordered secondary structure. Therefore, the spectra of the polymers at basic pH could, in principle, represent the CD signature of a (yet unknown) secondary structure of this novel type of polymers. Because the CD spectra of the basic samples remained unchanged, even when lowering the pH, the corresponding secondary structure is supposedly rather stable. An alternative explanation would invoke population of a random coil structure similar to an acidic environment, since the polymer spectra resemble each other in the far UV. The major difference, that is, the disappearance of the signal at 230 nm, could be explained by the loss of chirality transfer from the adjacent side chain to the triazole moiety, which, in acidic pH, might be mediated by an intramolecular hydrogen bond. A third explanation involving chemical decomposition is unlikely.

To gain further insight into the conformational behavior in solution, the pyrene-labeled polymers **10a$_{II}$** and **10b$_I$** were subjected to fluorescence spectroscopy studies. Please note that the conformational behavior of **10a$_{II}$** and **10b$_I$** is most likely affected by the presence of the pyrene labels and the different environment (vide infra) and hence caution should be taken by comparing them with the unlabeled polymers **7a$_I$** and **7b$_I$**. The poor solubility of both polymers **10a$_{II}$** and **10b$_I$** was one of the major obstacles for spectroscopic studies. It turned out that the polymers were only soluble in CH_2Cl_2 when small amounts of TFA were added (0.004 vol % for UV and CD and less than 0.0001 vol % for fluorescence measurements, corresponding to a ratio of monomer unit/TFA of approximately 1:25). During the course of the studies, it was found that TFE was a much better additive to dissolve the polymer than all other solvents used.

Exemplarily, the results of the spectroscopic measurements of the *all*-(L)-polypseudopeptide **10a$_{II}$** are summarized in Figure 7. The absorption spectra in various solvent mixtures show the characteristic vibronic fine structure of the attached pyrene moieties. These main electronic transitions are also reflected in the CD spectra, in particular, in neat CH_2Cl_2. The addition of large amounts of methanol lead to loss of CD intensity, and the CD signal completely vanishes when TFE is added. Solvent composition also affects the UV/vis spectra as methanol and, in particular, TFE addition lead to increased spectra resolution, pointing to decreased pyrene−pyrene ground-state interactions (preassociation). Additional pyrene−pyrene interactions

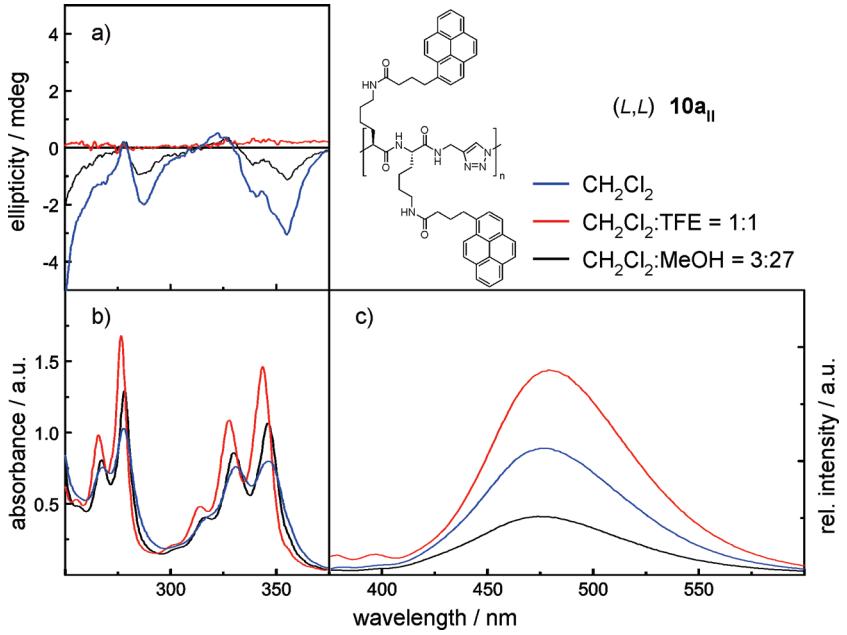


Figure 7. Overlay of (a) CD, (b) UV, and (c) fluorescence spectra of polymer **10a$_{II}$** in different solvents. Spectra were recorded in a 1 cm cuvette at 25 °C. Absorbance and CD spectra were measured at a concentration of 2.2×10^{-5} mol/L, fluorescence spectra were measured at a concentration of 6.8×10^{-7} mol/L at excitation wavelengths between 328 and 331 nm (depending on the respective UV absorption maximum of the sample).

involving one excited pyrene moiety lead to excimer formation characterized by broad and red-shifted emission bands. Such excimer formation is apparent from the emission spectra. Because of the high degree of side chain labeling and hence high local concentration of pyrene moieties, emission is almost exclusively arising from excimers,[17] and almost no monomer emission could be detected. However, careful inspection of the spectra reveals increased monomer emission (indicated by two weak maxima below 400 nm) in the case of methanol and, in particular, TFE addition. Therefore, all three optical spectroscopies point to a potentially folded structure in CH_2Cl_2, arranging the side-chain functionalities in close proximity. When methanol and TFE are present, a less-ordered and less-compact conformation seems to be populated. In comparison, the UV/vis and fluorescence emission spectra of the (D)-*alt*-(L)-polypseudopeptide **10b$_{I}$** (Figures S7 and S8 in the Supporting Information) display rather similar behavior, and all spectra (including CD) show a comparable trend with regard to solvent effects. However, the different backbone stereochemistry is clearly reflected in similar yet opposite signatures in the CD spectra (Figures S9 and S10 in the Supporting Information).

Summary and Outlook

By exploiting both the synthetic power and generated triazole amide-isostere in the "click" reaction, a new type of stereochemically well-defined polypseudopeptides was synthesized from readily available amino-acid-based monomers. Using stereochemically either (L,L) or alternating (D,L) dipeptide building blocks, our route provides facile access to strictly alternating polypseudopeptides, whose primary structure resembles naturally occurring channel-forming peptides (Gramicidins). Lysine-based polypseudopeptides of both (L,L)- and (D,L)-alternating stereochemistry were efficiently prepared using Cu-catalyzed 1,3-dipolar cycloaddition in the key polymerization step. Deprotection afforded water-soluble polymers with ionizable amino side chains, which could be quantitatively labeled with pyrene chromophores. The conformational behavior in solution was investigated using various optical techniques and the free polyamines display characteristic CD signals in aqueous solution depending on pH and side chain configuration. On the basis of the CD spectra and comparison to appropriate model compounds, the polyamines adopt a random coil structure in acidic pH, that is, when the side chains are protonated. When raising the pH, that is, when deprotonating the side chains, an irreversible transition to a yet unknown conformation takes place. The conformation of the fully pyrene-labeled polypseudopeptides was investigated in organic solvent mixtures, indicating a compact and folded structure in CH_2Cl_2, that is (at least partially), unfolded in the presence of polar additives (methanol, TFE), which supposedly break intramolecular hydrogen bonds. Although the obtained results indicate a rich conformational behavior of this new backbone type, no clear conformational assignments involving exact structures can be made. To determine these participating conformations in this new backbone type unequivocally, discrete oligomers with side-chain heterogeneity would have to be synthesized and then structurally characterized, in particular, by solution NMR techniques, as impressively demonstrated in the case of β-peptides.[18]

The incorporation of both triazole amide-isosteres and (D)-configured amino acid monomers in a precise backbone location constitutes the most advantageous feature of our newly developed polypseudopeptide family. This advantage enables fine-tuning of biodegradability and bioavailability, and hence we are currently looking into applying these polymers as scaffolds in various bioapplications.

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Supporting Information Available: Details of monomer synthesis and polymerization reactions as well as compound characterization data including UV/vis, CD, and NMR spectra

as well as chromatography. This material is available free of charge via the Internet at http://pubs.acs.org.

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