Synthesis and Characterization of a Telechelic Peptide as a Precursor for Supramolecular Networks

Stefania Federico,[1,2] *Andreas Lendlein,*[1,2] *Axel T. Neffe**[1,2]

Summary: A peptide showing propensity for the adoption of β-sheet conformation was synthesized in order to develop a defined molecular building block as part of a toolbox for the development of polymer networks based on supramolecular interactions. The peptide was synthesized by microwave-assisted solid phase peptide synthesis and the purification was performed by Reversed Phase-High Performance Liquid Chromatography (RP-HPLC), from which a purity higher than 95 mol-% was obtained. The calculated mass was confirmed by Matrix Assisted Laser Desorption Ionization-Time of Flight-Mass Spectrometry (MALDI-ToF-MS). Further characterization of the peptide was performed by IR and 2D TOCSY, NOESY, and HSQC NMR spectroscopy, which confirmed the identity and the sequence of the peptide.

Keywords: biomaterial; molecular recognition; peptides; supramolecular structures; telechelic precursor

Introduction

Peptides represent essential active molecules inside biological systems and consist of chains built from amino acids monomers.[1] Solid-phase peptide synthesis (SPPS)[2] is currently the most widely used method to synthesize peptides. Solid phase coupling offers the possibility for automization, ease of reagent separation, eliminate work-up steps during the synthesis and associated loss of product, and allows for high reaction yields due to excess reagents. Synthetic peptides and the physiologically more stable analogues, the peptidomimetics,[3,4] are generally used as bioactive compounds or lead structures and facilitate to study the relationships between chemical structure and biological activity. Recent examples are protease-sensitive peptides, which were investigated as part of a toolbox and precursor in the development of hydrogels.[5,6] Telechelic peptides or synthetic precursors can be used to develop polymer systems, which are families of closely related polymer-based materials with tailorable properties, in which small changes in the chemical composition significantly influence the macroscopic properties.[7,8] This approach was especially well studied for polymer networks. Polymer systems are demanded when tailoring the properties of a material to a certain application, e.g. in regenerative therapies.[9,10] Additionally, synthetic peptides and functionalized peptides have also been used in supramolecular chemistry, which aims at constructing highly complex, functional chemical systems from components held together by non-covalent intermolecular forces.[11,12] In particular, supramolecular interactions between peptides controlling the association of macromolecules have been used in synthetic systems employing the ability of the peptide to form β-sheets in solution.[13]

Here, the synthesis and characterization of the peptide LSELRLHNN, which was predicted to have some propensity to adopt extended β-chain conformation by

[1] Center for Biomaterial Development and Berlin-Brandenburg Center for Regenerative Therapies (BCRT), Institute of Polymer Research, Helmholtz-Zentrum Geesthacht, Kantstrasse 55, 14513 Teltow, Germany
E-mail: axel.neffe@hzg.de
[2] Institute of Chemistry, University of Potsdam, 14476 Potsdam-Golm, Germany

computer modeling, is discussed. It has to be noted that peptides ≤ 20 amino acids rarely adopt stable secondary conformations in diluted solutions, but that such conformation can be stabilized at higher concentration through intermolecular aggregation.[14,15] In order to employ such a functional peptide, it must be of high purity and needs to be carefully characterized to be able to draw structure-activity relationships. In the following, the solid-phase synthesis of the peptide LSELRLHNN is presented for its potential use as a telechelic precursor. The functional endgroups of the telechelics are the amino group and carboxylic acid at the end of the peptide backbone, which can be used for head-to-tail interactions and/or incorporation into networks. The HPLC purification of the peptide and its characterization by MALDI-ToF-MS, 2D TOCSY, NOESY, HSQC NMR spectroscopy and IR spectroscopy are discussed in detail.

Materials

N-Methylpyrrolidone (NMP), piperidine, acetonitrile, dichloromethane (CH_2Cl_2), and trifluoroacetic acid (TFA) were purchased from IRIS biotech (Marktredwitz, Germany). *N,N*-diisopropylethylamine (DIPEA) and acetic anhydride were purchased from Sigma-Aldrich (Munich, Germany). All Fmoc-protected amino acids, Fmoc-Asn-(Trt)-Wang resin, and Benzotriazole-1-yl-oxy-tris-pyrrolidino phosphonium hexafluorophosphate (PyBOP) were purchased from Novabiochem (Darmstadt, Germany). *Tert*-butyl methyl ether (TMBE) and triisopropylsilane (TIS) were purchased from Merck (Darmstadt, Germany). All chemicals were used as received.

Experimental Part

The peptide LSELRLHNN was synthesized on a LibertyTM Automated Microwave Peptide Synthesizer (CEM Corporation, Kamp-Lintfort, Germany) according to a 9-fluorenylmethoxycarbonyl (Fmoc) protocol using a Fmoc-Asn-(Trt)-Wang resin as solid support (substitution level = 0.7 mmol/g resin). The deprotection steps were performed with 20% (v/v) Piperidine in NMP solution. The coupling reactions were performed using 5 eq of PyBOP in NMP (0.5 M), 5 eq of amino acids in NMP (0.2 M), and 10 eq of DIPEA in NMP solution (2 M). Each deprotection and coupling reaction was performed with microwave energy (75 °C) and nitrogen bubbling. Each coupling was followed by a capping step with 10% (v/v) acetic anhydride in NMP (except for the N-terminal end). The dried resin was manually cleaved using a solution of 95% TFA, 2.5% H_2O, and 2.5% (v/v) TIS. The reaction was allowed to proceed for 90 min with occasional shaking. The peptide was precipitated with *tert*-butyl methyl ether (TBME) and collected by centrifugation (20 min, 3000 rpm, 4 °C). The final product was dissolved in water and lyophilized.

RP HPLC: The purification of the peptide was performed on a Varian HPLC system (Prostar, Model 701) by using a PS/DVB reversed-phase semi-preparative column (PLRP-S, pore size: 100 Å, 8 μm; 300 * 25 mm). Each purification run was carried out with a linear gradient of water (0.1% (v/v) TFA, buffer A) and acetonitrile (0.1% (v/v) TFA, buffer B) from 10% to 90% B in 50 min at a flow rate of 9 mL/min. A wavelength of 220 nm was used for the detection of the peptide.

MALDI-ToF-MS: Matrix-Assisted Laser Desorption-Ionization Time-of-Flight Mass Spectrometry was performed on a Bruker (Bremen, Germany) Autoflex III Smartbeam MALDI-ToF mass spectrometer. The instrument was working in Reflector Mode with an acceleration voltage of 20 kV and a UV laser working at 355 nm with a rate of 200 Hz. For a single mass spectrum 2000 shots (4×500) were accumulated. Alpha-Cyano-4-hydroxycinnamic acid was used as matrix.

FTIR: Fourier Transform Infrared spectra were recorded on a Shimadzu FTIR spectrometer (Model 8400S). The peptide was investigated in the lyophilized form.

NMR: Nuclear Magnetic Resonance spectra were recorded on a NMR Bruker system (DRX 500 Avance; software Topspin version 1.3).

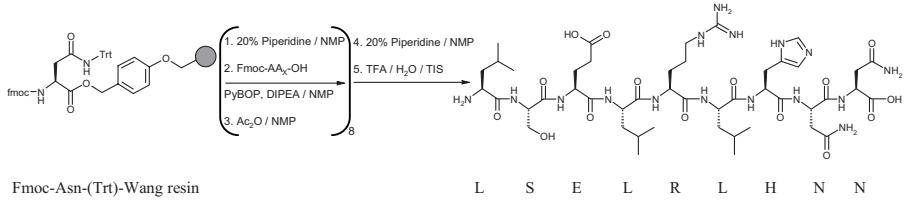
Results and Discussion

Peptide Synthesis

Automated, microwave assisted peptide synthesis consisted in the repeating steps deprotection of the amino terminus, coupling of the next amino acid, and capping of the unreacted amino groups (Scheme 1) for the synthesis of the nonapeptide LSELRLHNN.

The choice of protecting group, solvent, solid support, coupling agent, and reagent conditions is thereby important for the yield and purity of the product. These parameters will be discussed in more detail in the following for the synthesized peptide.

The Fmoc-strategy for the peptide synthesis was chosen because the reaction conditions and cleavage are much milder compared to the Boc strategy, resulting in higher yields and purer compounds. In the Fmoc protocol, coupling steps are performed under neutral or slightly basic conditions, amino group deprotection is performed under moderate alkaline conditions, and side chain protection as well as cleavage from the resin is performed under acidic conditions.[16] One special point of the deprotection is the release of a dibenzofulvene which can be quantified by UV spectroscopy in each coupling step.[17] Classically, DMF was the standard solvent for peptide synthesis, however, NMP does not have the DMF inherent toxicity and was therefore preferred. The coupling steps require activation of the free carboxylic acid groups. Classical activators such as carbodiimides often result in too low coupling yields and might lead to racemization of some amino acids. PyBOP does not suffer from these drawbacks and the higher solubility of this hexafluorophosphate salt compared to tetrafluoroborate salts allows for the preparation of stable and highly concentrated solutions.[18] While the chemical activation of the carboxylic acid groups enables reaction at room temperature, peptide synthesis with microwave irradiation is more efficient and often requires fewer equivalents of amino acids and coupling reagents compared to conventional synthesis. Typical solid supports used for solid-phase synthesis consist of polystyrene with a 1–2% DVB crosslinking. The Wang resin, resulting from the addition of the *p*-alkoxybenzyl alcohol linker to the polystyrene, is a typical support for the synthesis of peptide with free carboxyl acid groups on the C-terminus. For the described synthesis a pre-loaded resin with the amino acid Asparagine was used in order to increase the yield of the reaction. In general, during peptide synthesis, accumulation of deletion sequences, especially those missing a single residue, are frequent, resulting in reduced yields and difficult purification of the desired full-length product. Therefore, acetic anhydride was used



Scheme 1.

Steps in solid phase peptide synthesis using Fmoc-chemistry. Solid support: Fmoc-Asn-(Trt)-Wang resin; 1. deprotection: 20% (v/v) Piperidine in NMP; 2. coupling: Fmoc-AA$_x$-OH, PyBOP and DIPEA in NMP; 3. capping: 10% (v/v) Acetic anhydride in NMP; 4. deprotection of the N-terminal end: 20% (v/v) Piperidine in NMP; 5. deprotection of the side chains and cleavage from the resin: 95% TFA/2,5% H$_2$O/2,5% TIS (v/v).

as capping reagent in order to stop the growing of unwanted sequences due to failed couplings. Finally, the goal of the final cleavage and deprotection was to separate the peptide from the support while removing the protecting groups from the side-chains. This reaction was allowed to proceed for 90 min in order to minimize the exposure of the peptide to the cleavage reagents. Prior to the cleavage reaction, the resin was swollen in CH_2Cl_2 and dried in order to remove any trace of NMP, which could cause reduction of the yield. Triisopropylsilane, which is a nucleophilic scavenger, was used in the cleavage solution in order to quench the reactive cationic species, which are generated from the cleavage of the side chains protecting group. A good example for such side reaction is the protecting group of the Arginine side chain (Pbf) which is highly reactive but easily scavenged.[19]

In order to induce the peptide precipitation, the cleavage mixture was added dropwise to *tert*-butylmethylether so that the more lipophilic by-products remained in solution. The precipitate was then recovered by centrifugation, dissolved in water, and lyophilized.

RP-HPLC Purification and MALDI-ToF-MS Characterization

RP-HPLC is the most powerful and rapid method to purify peptides through adsorption/desorption of the desired peptide and side products to the hydrophobic stationary phase. Often, C_{18} modified silica is used as column material, however, the used polymer solid phase (PS/DVB) has the advantageous properties to operate over the entire pH range, enabling reproducible resolution with greatly extended lifetimes. Since the peptide LSELRLHNN is moderately hydrophilic, a linear gradient was used, starting from 10 vol% of organic phase (acetonitrile, buffer B) until 90 vol% over a time of 50 min. The peptide was dissolved in the same mixture of the initial run conditions (10 vol% acetonitrile – 90 vol% water). Using these conditions the elution time of the peptide was around 25 min. The method efficiently purified peptides that have a similar length and amino acids composition as the studied peptide (data not shown).

After purification, the received mass of the peptide was 411 mg corresponding to a yield of 37.5 mol-% of isolated product (88 mol-% per coupling step). The purity of the peptide was greater then 95 mol-% as determined by RP-HPLC after injection of the pure peptide.

The mass of the peptide was confirmed by MALDI-ToF-MS. From the MALDI-ToF mass spectrum of the peptide LSELRLHNN it was possible to identify only one peak corresponding to the mass of

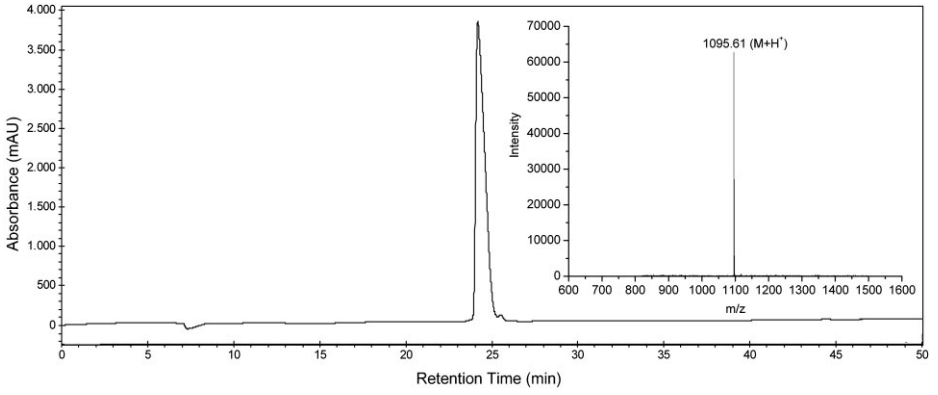


Figure 1.
RP-HPLC chromatogram of the peptide LSELRLHNN (purity > 95 mol-%). Linear gradient in water and acetonitrile (0.1 vol% TFA). Right insertion: MALDI-ToF-MS spectrum of the peptide. Calculated mass: 1094.58; found ($M + H^+$): 1095.61.

the peptide plus H^+ ions. This was a further confirmation that highly pure peptide was isolated (Figure 1).

FTIR Characterization

A further investigation of the peptide structure was performed by FTIR spectroscopy, which is widely used to characterize the secondary structure of peptides.[20] The amide I band, which is mainly associated to the C=O stretching vibration of the polypeptide backbone, is the most prominent and is found in the range 1600–1700 cm^{-1}. In the FTIR spectrum of the peptide LSELRLHNN (Figure 2) two bands were found at 1663 cm^{-1} and 1624 cm^{-1}. These values corresponds to the values for turn like peptides ($1664 \pm 2\,cm^{-1}$)[21] and extended chains, which indicates no overall defined secondary structure. A second major band in a peptide backbone is the amide II, which results from the N-H bending vibration and from the C-N stretching vibration (1535 cm^{-1}). The peak at 1425 cm^{-1} was assigned to C-O, COO-stretching vibration. The peaks at 3272 cm^{-1} and 3082 cm^{-1} were assigned to OH stretching vibrations and NH bending, while the band at 2961 cm^{-1} is characteristic for C-H vibrations.

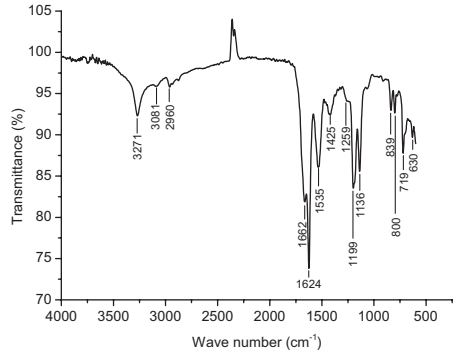


Figure 2.
FTIR spectrum of the peptide LSELRLHNN.

2D NMR Characterization

Because of the large number of protons per residue and the resulting resonance overlap even small peptides cannot be fully characterized from one dimensional spectra and require two dimensional analyses. Therefore the solution conformation of the peptide LSELRLHNN was investigated by 2D NMR. In order to prevent loss of signals of amide protons from exchange with solvent deuterons, the peptide was measured in 90 vol% H_2O/10 vol% D_2O at pH = 3. Solvent suppression for the analyses was achieved using the WATERGATE pulse sequence. Detailed investigations of the peptide structure were performed by TOCSY (Total Correlation Spectroscopy), NOESY (Nuclear Overhauser Enhancement Spectroscopy), and HSQC (Heteronuclear Single Quantum Correlation) and a full sequential assignment of all spectral peaks was achieved. In the TOCSY spectrum, spins are correlated by scalar couplings, whose magnitude depends on the number of intervening bonds, while the NOESY spectrum contains information about spatial proximities

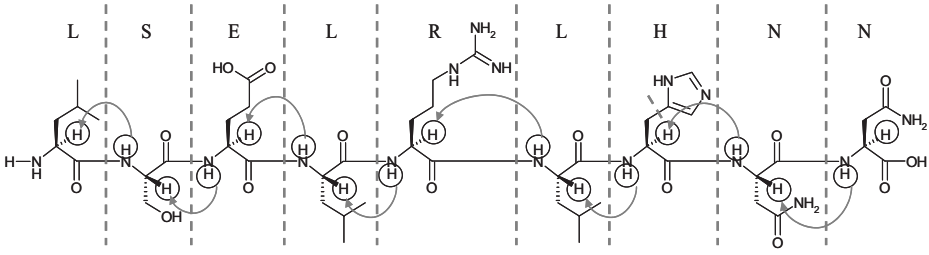


Figure 3.
Schematic representation of the spin systems of the peptide LSELRLHNN correlated by scalar couplings (dashed lines) and dipolar couplings (arrows).

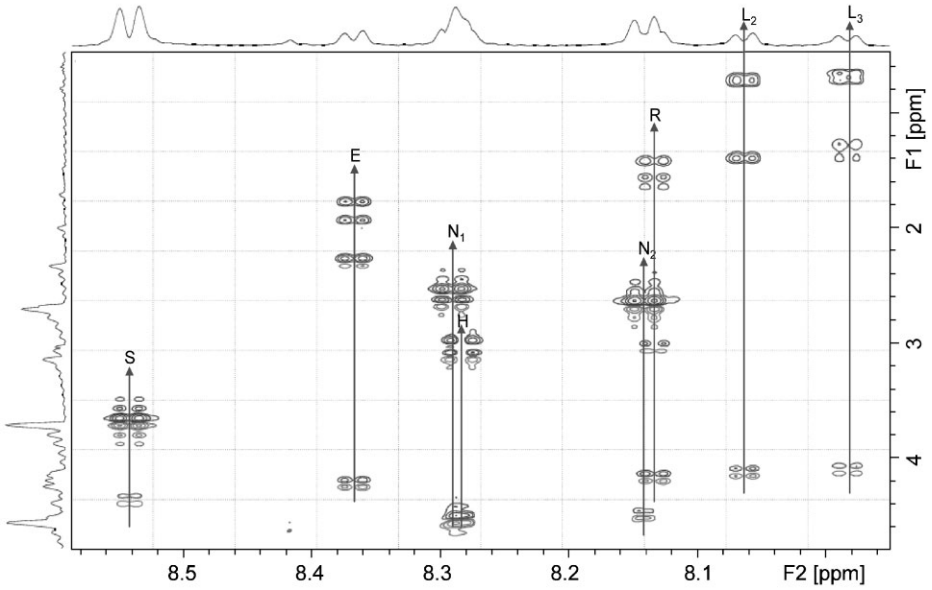


Figure 4.
N_H region of the TOCSY spectrum of the peptide LSELRLHNN.

and the spins are correlated by dipolar couplings resulting from the interactions of spins through space and therefore only depend on the distance and not on the number of intervening bonds.[22] Figure 3 is a schematic representation of the peptide sequence with the respective spin systems (dashed lines) and dipolar couplings (arrows). In the TOCSY spectrum, the assignment of the well separated NH traces (Figure 4) was carried out, whereby all protons in the same spin system as the backbone NH were found. The first leucine (L_1) residue could not be assigned from this region of the spectrum as the protons of the free amino group have a different chemical shift and as the signal is not as strong because of proton exchange with the solvent. However, the peaks could be identified from the CH_α region. The proton chemical shifts of the amino acids were assigned by comparison to the *Biological Magnetic Resonance Data Bank* (Table 1).

From the analysis of the NOESY spectrum the sequential correlation of the amino acids present in the peptide was confirmed (Figure 5). As discussed for the TOCSY spectrum, also in the N_H region of the NOESY it was difficult to identify the crosspeak between the serine (S) and the leucine (L_1) residues. Additionally to the sequence structure, 2D NOE spectra can be used to determine defined secondary structures of peptides in solution such as turns and helices, as in this case additional crosspeaks from protons close in space (generally distance <5 Å) would occur in the spectrum. Such peaks were not observed here, so that no indication of a defined secondary structure was found.

Table 1.
^{1}H chemical shifts of the peptide assigned from the TOCSY spectrum.

	N_H	H_α	H_β	H_γ	H_δ	H_ε
L_1	8.05	4.09	1.39	1.39	0.70	
S	8.54	4.39	3.65			
E	8.37	4.20	1.93–1.77	2.26		
L_2	8.07	4.10	1.39	1.39	0.72	
R	8.14	4.13	1.55	1.41	3.01	
L_3	7.99	4.07	1.39	1.27	0.70	
H	8.29	4.54	3.08–2.98		8.49	7.16
N_1	8.28	4.50	2.62–2.53			
N_2	8.15	4.52	2.63			

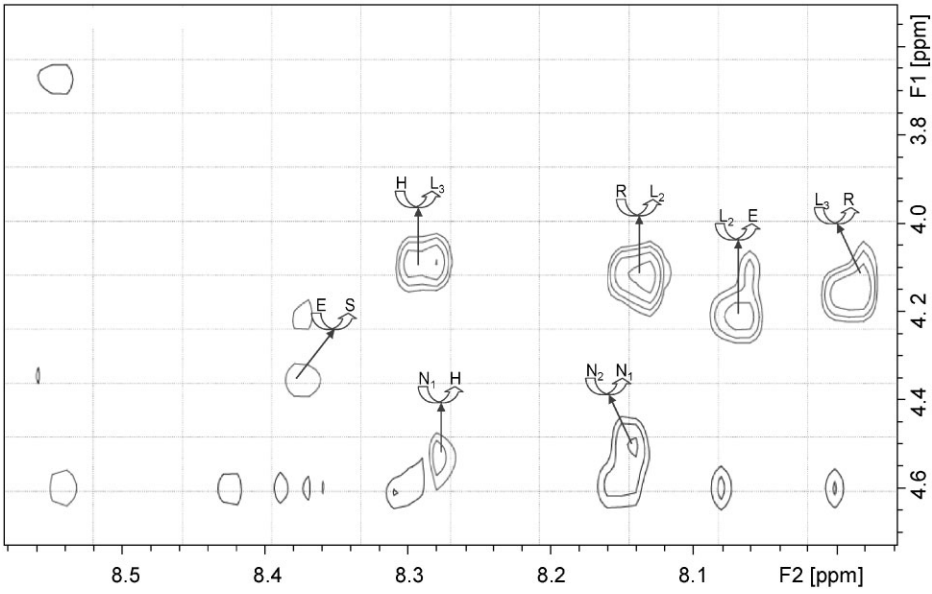


Figure 5.
N_H region of the NOESY spectrum of the peptide LSELRLHNN.

From the analysis of the HSQC spectrum it was possible to identify most of the protons with their directly bound carbon atoms (Figure 6). In particular it was possible to identify even the protons H_δ and H_ε (with their respective carbon atoms) of the imidazole functional group of the histidine which were not identified in the

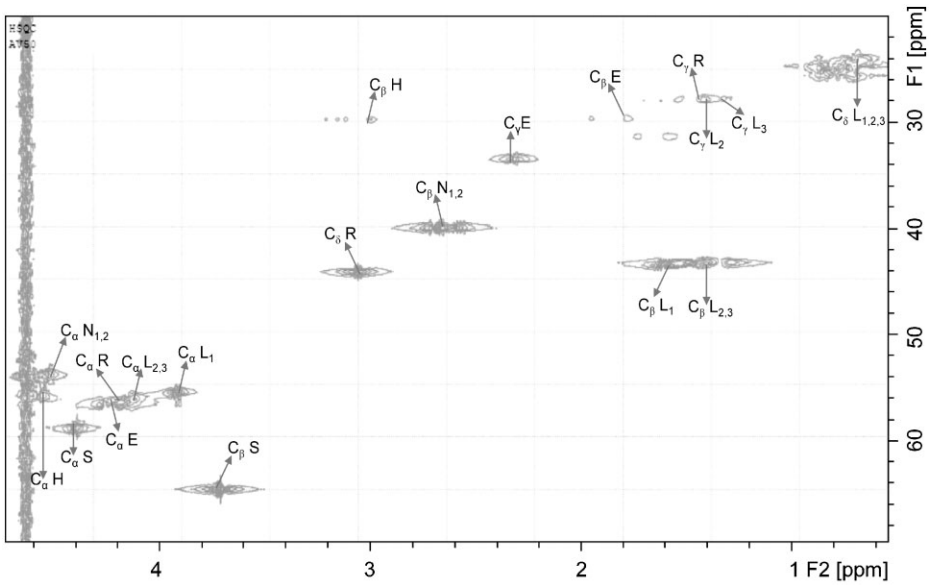


Figure 6.
HSQC spectrum of the peptide LSELRLHNN.

Table 2.
^{13}C chemical shifts of the peptide assigned from the HSQC spectrum.

	C_α	C_β	C_γ	C_δ	C_ε
L_1	55.98	43.17	27.72	24.73	
S	58.78	64.58			
E	56.36	29.61	33.52		
L_2	55.98	43.17	27.72	24.73	
R	56.30	31.27	27.84	44.04	
L_3	55.98	43.17	27.72	24.73	
H	55.76	29.81		120.6	137.7
N	53.98	39.8			
N	53.49	39.8			

TOCSY spectrum. All chemical shifts assigned in the HSQC spectrum are summarized in Table 2.

Conclusion

The synthesis of a leucine-rich repeat peptide (LSELRLHNN) was successfully carried out and was obtained with a purity higher than 95 mol-% as shown by RP-HPLC. A detailed investigation by FTIR, TOCSY, NOESY, and HSQC spectroscopy was performed in order to confirm the order of the amino acids in the sequence and the structural properties of the peptide. Due to the high purity and binding potential of the designed peptide to ECM macromolecules, this specific sequence can be considered as a promising telechelic building block for the development of supramolecular polymer systems based on non-covalent interactions similar to those present in biological processes.

[1] D. Chow, M. L. Nunalee, D. W. Lim, A. J. Simnick, A. Chilkoti, *Mat. Sci. Eng. R.* **2008**, *62*, 125.
[2] J. M. Stewart, *Int. J. Peptide Res. Ther.* **2007**, *13*, 3.
[3] A. T. Neffe, B. Meyer, *Angew. Chem. Int. Ed.* **2004**, *43*, 2937.
[4] A. T. Neffe, M. Bilang, B. Meyer, *Org. Biomol. Chem.* **2006**, *4*, 3259.
[5] G. P. Raeber, M. P. Lutolf, J. A. Hubbell, *Biophys. J.* **2005**, *89*, 1374.
[6] M. P. Lutolf, J. A. Hubbell, *Nat. Biotech.* **2005**, *23*, 47.
[7] Y. K. Feng, M. Behl, S. Kelch, A. Lendlein, *Macromol. Biosci.* **2009**, *9*, 45.
[8] A. Lendlein, P. Neuenschwander, U. W. Suter, *Macromol. Chem. Phys.* **2000**, *201*, 1067.
[9] A. Maslovskis, N. Tirelli, A. Saiani, A. F. Miller, *Soft Matter* **2011**, *7*, 6025.
[10] V. P. Shastri, A. Lendlein, *Adv. Mater.* **2009**, *21*, 3231.
[11] A. T. Neffe, A. Zaupa, B. F. Pierce, D. Hofmann, A. Lendlein, *Macromol. Rapid Comm.* **2010**, *31*, 1534.
[12] P. Y. W. Dankers, M. C. Harmsen, L. A. Brouwer, M. J. A. V. Luyn, E. W. Meijer, *Nat. Mater.* **2005**, *4*, 568.
[13] A. N. Elder, N. M. Dangelo, S. C. Kim, N. R. Washburn, *Biomacromolecules* **2011**, *12*, 2610.
[14] A. G. Fort, D. C. Spray, *Biopolymers* **2009**, *92*, 173.
[15] J. K. Myers, C. N. Pace, J. M. Scholtz, *Biochemistry* **1997**, *36*, 10923.
[16] D. Orain, J. Ellard, M. Bradley, *J. Comb. Chem.* **2002**, *4*, 1.
[17] W. S. Newcomb, T. L. Deegan, W. Miller, J. J. A. Porco, *Biotech. Bioeng.* **1998**, *61*, 55.
[18] T. Bruckdorfer, O. Marder, F. Albericio, *Curr. Pharm. Biotech.* **2004**, *5*, 29.
[19] L. A. Carpino, H. Shroff, S. A. Triolo, E. M. E. Mansour, H. Wenschuh, F. Albericio, *Tetrahedron Lett.* **1993**, *34*, 7829.
[20] S. Luo, C. F. Huang, J. F. McClelland, D. J. Graves, *Anal. Biochem.* **1994**, *216*, 67.
[21] D. M. Byler, H. Susi, *Biopolymers* **1986**, *25*, 469.
[22] A. Bax, *Annu. Rev. Biochem.* **1989**, *58*, 223.

