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pH-sensitivity and Conformation Change of the N-terminal Methacrylated Peptide VK20

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ABSTRACT

N-terminal methacrylation of peptide MAX1, which is capable of conformational changes by variation of the pH, results in a peptide, named VK20. Increasing the reactivity of this terminal group enables further coupling reactions or chemical modifications of the peptide. However, this end group functionalization may influence the ability of conformational changes of VK20, as well as its properties. In this paper, the influence of pH on the transition between random coil and β -sheet conformation of VK20, including the transition kinetics, were investigated. At pH values of 9 and higher, the kinetics of β -sheet formation increased for VK20, compared to MAX1. The self-assembly into β -sheets recognized by the formation of a physically crosslinked gel was furthermore indicated by a significant increase of G' . An increase in pH (from 9 to 9.5) led to a faster gelation of the peptide VK20. Simultaneously, G' was increased from 460 ± 70 Pa (at pH 9) to 1520 ± 180 Pa (at pH 9.5). At the nanoscale, the gel showed a highly interconnected fibrillary network structure with uniform fibril widths of approximately 3.4 ± 0.5 nm ($N=30$). The recovery of the peptide conformation back to random coil resulted in the dissolution of the gel, whereby the kinetics of the recovery depended on the pH. Conclusively, the ability of MAX1 to undergo conformational changes was not affected by N-terminal methacrylation whereas the kinetics of pH-sensitive β -sheet formations has been increased.

INTRODUCTION

In nature, specific functions are influenced just by small structural changes in the protein conformation. Inspired by this, peptides have been extensively exploited as building blocks for stimuli-responsive materials [1] with focus on the basic conformational motifs, such as α -helix, β -sheet or coiled coil as structural elements. These conformational transitions were dynamic in response to the conditions of the environment, e.g. change of pH, salt, and temperature or

presence of enzymes [2]. The integration of the peptide moieties into other materials enabled the control of their molecular organization e.g. assembly and disassembly of peptide-polymer conjugates [3] or peptide coated gold nanoparticles [4]. In some cases, the responsiveness on the molecular level resulted in macroscopic changes of the material properties, e.g. sol-gel transitions or volume changes in hydrogels. Regarding the integration of peptide moieties into other materials, the ability for hydrogen bonding and the flexibility of the peptides have to be maintained to allow the peptide to undergo conformational changes [5, 6]. Due to their easier synthetic accessibility compared to the relatively long α -helix/coiled coil peptides, β -sheets have attracted enormous interest. One of the reported β -hairpin system is MAX1 (VKVKVKVKVpPTKVKVKV-NH₂) [7, 8], a peptide consisting of two strands of alternating valine and lysine residues connected by a central tetra-peptide adopting a β -turn. MAX1 is a promising candidate for studies on the change of conformation of peptides as it can undergo pH-triggered β -sheet formations accompanied by a sol-gel transition. At neutral or acidic pH, the eight lysine moieties and the amine at the N-terminal are protonated (with a net charge of +9), resulting in electrostatic repulsion, which prevents the folding of the peptide MAX1. Once the pH is increased to 9, the peptide adopts a β -hairpin structure. Subsequent self-assembly creates temporary crosslinks resulting in hydrogel formation. The functionalization of the peptide MAX1 with reactive groups enables its coupling to macromolecules or additional chemical modifications. However, direct modification of peptides e.g. N-terminal, C-terminal or at side chains, might alter their properties including hydrophobicity, charges, and steric demands [9-11]. This can influence the ability for conformational changes and in some cases cause the loss of those transitions. Therefore, investigations on the stimuli-sensitivity after introducing a reactive group directly at the terminal are of great importance. In this work, we explored whether a methacrylated MAX1, named VK20, can be used as a building block for the fabrication of stimuli-sensitive peptide materials. The ability of the modified peptide VK20 to undergo conformational changes was investigated as a function of pH value. In addition, the transition kinetics between random coil and β -sheet, as well as the pH-sensitive self-assembly of VK20, were studied.

EXPERIMENT

Acetic acid, sodium acetate, 2-*N*-morpholino ethanesulfonic acid (MES), tris-hydroxymethyl aminomethane (Tris), bis-tris propane (BTP), boric acid, D₂O, NaOD (40 wt% D₂O solution), DCl (35 wt% D₂O solution), trifluoroacetic acid (TFA), acetonitrile, uranyl acetate, and congo red were all purchased from Sigma-Aldrich (Taufkirchen, Germany). Buffer solutions with pH ranging between 5 and 9.5 were prepared as followed: pH 5 (acetate), pH 6 (MES), pH 6.5 (MES), pH 7 (Tris), pH 8 (BTP), pH 8.5 (boric acid), pH 9 (boric acid), and pH 9.5 (boric acid). VK20 was purchased from Smartox Biotechnology (Saint Martin d'Hères, France). The purity of VK20 was determined by means of High Performance Liquid Chromatography (HPLC) (Agilent 1200, Waldbronn, Germany) using a reversed-phase column (RP-18, pore size: 100 Å, 5 µm; 125*4 mm) by detecting the signals at 220 nm. The experiment was carried out with a linear gradient of buffer A (0.1 vol% TFA in H₂O) and buffer B (0.1 vol% TFA in acetonitrile) from 5 vol% B to 95 vol% B for 35 min at a flow rate of 1 ml·min⁻¹. Matrix-assisted Laser Desorption Ionization-Time of Flight (MALDI-TOF) mass spectrometry was performed on a Bruker (Bremen, Germany) Autoflex III Smartbeam mass spectrometer.

¹H-NMR spectra of VK20 were recorded at 25 °C in D₂O with a Mercury 500 spectrometer (Bruker, Karlsruhe, Germany). Concentrated DCl and NaOD solution were used to adjust the pH values.

Fourier-transform infrared (FTIR) spectroscopy was performed on a Nicolet IR 6700 spectrometer equipped with an attenuated total reflectance (ATR) module. Peptide solutions were prepared by dissolving the deuteriochloride salt of solid VK20 in D₂O, resulting in a 1 wt% solution. Concentrated NaOD was added to increase the pH whereby the sample gelled. Afterwards concentrated DCl was used to decrease the pH resulting in dissolution of the gel. Deuterated VK20 * nDCl was prepared by lyophilizing the TFA salt of VK20 from 0.1 M DCl.

Ultraviolet Visible (UV-VIS) absorption spectra were recorded with a Cary 100 spectrophotometer (Agilent, Darmstadt, Germany) at ambient temperature (20 °C). Samples were measured in solution in a cuvette with 1 cm path length. Absorption spectra were recorded in the range of 300-700 nm.

Circular Dichroism (CD) spectra were collected on a J-1500 spectropolarimeter (Jasco, Hachioji, Japan). An equal volume of VK20 solution and the corresponding buffer solutions were mixed first to yield the final VK20 solution and then transferred to a 1 mm quartz glass cell. The temperature was kept constant at 25 °C.

Rheological investigations were performed on a Haake Rheowin Mars III (Thermo Scientific, Karlsruhe, Germany) by using 20 mm plate-plate geometry at 25 °C. An equal volume of the peptide solution and the corresponding buffer solution were mixed to form the desired solution and then transferred to the rheometer for time sweep measurements at constant stain of 0.1% and a frequency of 0.1 Hz.

Bright field images of the VK20 gel were taken on a CM12 transmission electron microscope (TEM) (Philips, Eindhoven, Netherlands). 1 wt% of VK20 solution was incubated at pH 9 for 24 h to allow complete gelation prior to sample preparation. The gel was diluted 20 times with deionized water. A 5 µL aliquot of diluted gel was placed on a 400 mesh copper grid. The sample was then negatively stained by pipetting a drop of 2% (w/v) uranyl acetate aqueous solution on the grid and was subsequently left to dry. The fibril width measurements were carried out using the software Image J.

RESULTS and DISCUSSION

The properties of the methacrylate functionalized peptide VK20 were investigated by various methods. The purity, which was determined by HPLC, was above 95%. The identity of VK20 (Figure 1) was confirmed by MALDI-TOF observing a molecule peak at $m/z= 2298.04$ Da ($C_{111}H_{202}N_{29}O_{22}$) and the corresponding sodium ion at $m/z= 2320$ Da ($C_{111}H_{202}N_{29}O_{22}[Na^+]$).

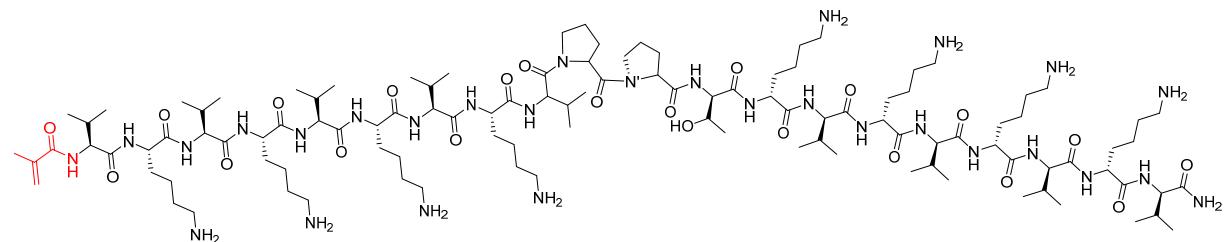


Figure 1 Chemical structure of peptide VK20

CD spectroscopy was applied for investigations on the pH-sensitivity of the conformational changes of VK20. Therefore, buffer solutions with pH values varying between 5 and 9 were mixed with VK20 solutions (0.05 wt%). Below pH 9, VK20 exhibited a negative peak at 198

nm, attributed to a random coil structure. After addition of pH 9 buffer solution, the CD spectrum of VK20 displayed a very clear minimum at 216 nm indicating the presence of β -sheets (Figure 2a) [7, 12]. The mechanism of conformational change can be explained as follows: at a pH below 9, the eight lysine moieties are protonated, which causes electrostatic repulsion preventing the folding of VK20. When the pH was increased to 9, the formation of β -sheet was triggered by the deprotonation of lysine moieties that lowered the electrostatic repulsion and subsequently dominated intramolecular hydrophobic interaction and hydrogen bonding.

The pH-induced reversible conformation transition of VK20 was further studied by FTIR spectroscopy. The IR spectra of a 1 wt% VK20 solution in D₂O (pH 4.5) displayed an amide I band at 1644 cm⁻¹ indicating a random coil conformation of VK20 (Figure 2b). This observation was similar to the reported unordered conformation of peptides displaying an amide I band at 1645 cm⁻¹. An increase of the pH to 9.2 by addition of NaOD caused gelation of the mixture and a shift of the amide I band to 1618 cm⁻¹ was observed, indicating the presence of β -sheets in the structure of VK20 [7]. This downshift could be attributed to the formation of hydrogen bonds resulting in the arrangement into a β -sheet structure, which affected the amide vibrations in the peptide backbone [13]. This is in consistency of reported β -sheet structures [14]. In addition, a weak band at 1680 cm⁻¹ was observed, which may indicate an antiparallel arrangement of the β -sheets or a β -turn structure. The observed β -sheet band below 1620 cm⁻¹ could be attributed to β -sheet rich aggregates. When the pH was lowered again to an acidic pH of 5.5 by adding a small amount of DCl, the physically crosslinked gel dissolved attributed to the recovery of the random coil conformation, suggesting the reversibility of this transition.

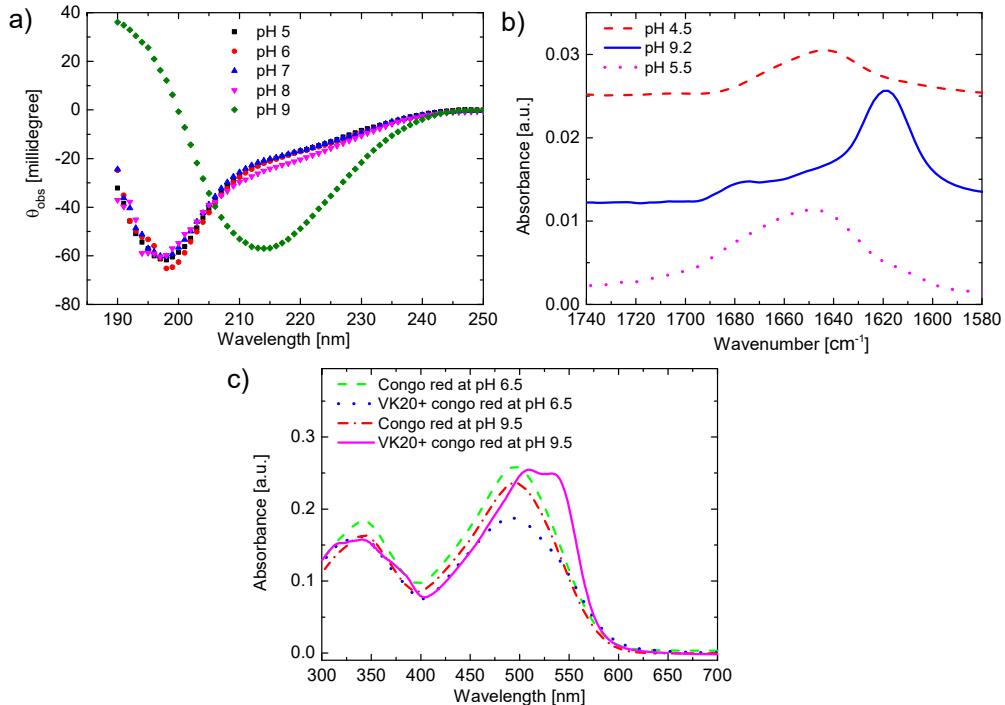


Figure 2 Characterization of the conformations of VK20 solutions by CD spectroscopy, IR spectroscopy and UV spectroscopy (Congo red binding study). a) CD spectra of 0.05 wt% VK20 solution in different pH buffer solutions (pH 5: black square, pH 6: red circle, pH 7: blue up triangle, pH 8: purple down triangle, pH 9: green diamond); b) Amide I band IR spectra: 1 wt% VK20 D₂O solution (red dashed line); after addition of concentrated NaOD gelation occurred (blue solid line); dissolved gel after addition of concentrated DCl (purple dotted line); c) UV spectra of 0.005 wt% VK20 solution in pH 6.5 and 9.5 (Congo red in pH 6.5: green dashed line, VK20 with Congo red in pH 6.5: blue dotted line; Congo red in pH 9.5: red dash-dotted line, VK20 with Congo red in pH 9.5: purple solid line).

A specific dye binding study using Congo red was performed to further confirm the existence of a β -sheet structure of VK20 at basic pH. Congo red is a frequently used dye for examining the cross- β -sheet structure in amyloid-like proteins, as it binds specifically to β -sheet regions of the peptides [15]. Once the dye is bound at the β -sheet structure, the orientation of dye molecules can be analyzed by UV spectroscopy. In Figure 2c the UV spectra of Congo red with and without diluted VK20 solution at pH 6.5 and pH 9.5 is shown. For Congo red without peptide two maxima absorption peaks at 340 and 490 nm were observed, which were independent of the pH. After addition of the peptide, a pH dependent UV absorption of the dye was observed. At pH 9.5, the dye showed a shift of the second maximum from 490 nm to 540 nm indicating the binding of the dye with β -sheet structures whereas the maximum absorption peak

of Congo red in VK20 solution at pH 6.5 was unaffected. These results further provided an additional hint for the existence of β -sheet structures within VK20 gel at basic pH.

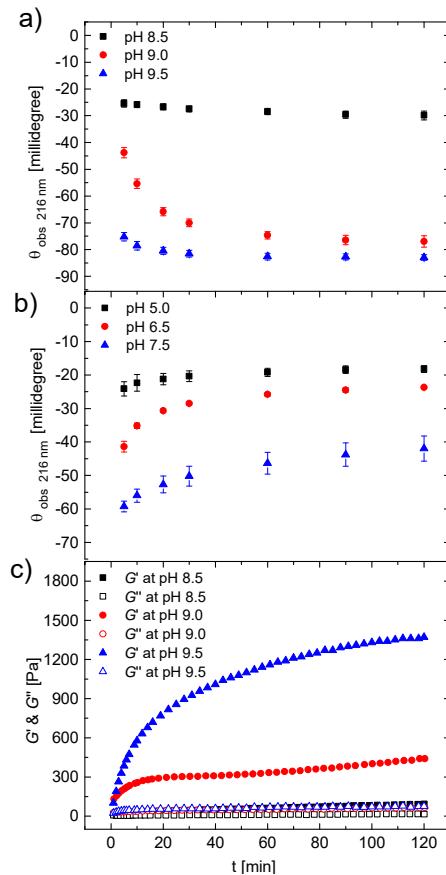


Figure 3 Kinetic study of conformation transition of VK20 peptide solution a) Rates of β -sheet formation for 0.05 wt% solutions of VK20 monitored by measuring the ellipticities at 216 nm ($\theta_{\text{obs}} \text{ 216 nm}$) as a function of time at pH 8.5 (black square), pH 9 (red circle), pH 9.5 (blue triangle); b) Rates of β -sheet returning to random coil for 0.05 wt% VK20 solution after mixing with pH 9.5 buffer solution monitored by measuring $\theta_{\text{obs}} \text{ 216 nm}$ as a function of time at pH 5.0 (black square), pH 6.5 (red circle), pH 7.5 (blue triangle); c) Time sweep rheological measurements of 1 wt% VK20 solution at pH 8.5 (G' black solid square, G'' black open square), pH 9.0 (G' red solid circle, G'' red open circle), pH 9.5 (G' blue solid triangle, G'' blue open triangle).

The kinetics of the conformational change of the peptide depending on the pH value was investigated by CD spectroscopy (Figure 3). The ellipticities of VK20 at pH 8.5 remained constant over time whereas they decreased at pH 9 and 9.5. This observation was more significant at pH 9.5. This effect was attributed to the pH-dependent transition between a random coil and β -sheet conformation. At pH 8.5 VK20 in general still adopts a random coil conformation followed by a transition to β -sheets at pH 9 and 9.5 within 90 min and 30 min,

respectively. This observation indicated a pH-dependent kinetics of β -sheet formation. Interestingly, the rates of β -sheet formation are much faster than those reported for the peptide MAX1 at a similar concentration [7]. This can be attributed to methacrylate group at the N-terminal that decreased the net charge of VK20 and increased the hydrophobicity of the peptide resulting in faster changes conformation. In addition to studies on the formation of β -sheets, the pH-dependent recovery of the conformation from β -sheet back to random coil was investigated. The pH of VK20 solutions at pH 9.5 was decreased to pH 7.5, 6.5 and 5, respectively, and the ellipticities were recorded as a function of time. At pH 7.5, the ellipticities reached a plateau after 120 min, and after 60 min at pH 6.5. When the pH was decreased to 5.0, a stable plateau of ellipticities was reached within 30 minutes, indicating the transition into β -sheets was completed. These results suggested that the kinetics of conformational change could be tuned by adjusting the pH.

In addition to the kinetic studies of the transition by CD spectroscopy, rheological measurements were performed. Here, the storage modulus G' and the loss modulus G'' can be determined, which correlate to the number of netpoints in the system. If the peptide would adopt a random coil structure, no netpoints would be present, obtaining a solution of the peptide. In contrast, β -sheet formation would generate netpoints. Therefore an increase in G' would be expected to be recognized by a gelation of the peptide. The time sweep rheological experiments of 1 wt% peptide solution in boric buffer solution of pH 9 and 9.5 at constant strain and constant frequency showed a significant increase of the storage modulus G' compared to a solution at pH 8.5, indicating the self-assembly of peptide sequences into physically gelled polymer networks (Figure 3c). Furthermore, the peptide solution at pH 9.5 reached a G' of 650 ± 50 Pa within 10 min and having a final plateau of final G' was 1520 ± 180 Pa at 120 min, whereas the solution at pH 9 showed slower kinetics with a plateau of G' at 460 ± 70 Pa after 120 min. The storage modulus of the resulting VK20 gel at pH 9.5 was similar to or larger than several reported physically gel systems with a G' between 100 and 1000 Pa [16, 17].

The self-assembly induced by the conformational change of VK20 was further investigated by NMR spectroscopy, a valuable method for the characterization of self-assembly by determining the differences in mobility of molecules in solution and gel states [18]. As shown in the ^1H NMR spectra of VK20 at a pH of 4.5 (Figure 4a), the signals of the double bond protons in the methacrylate appeared at 5.69 and 5.50 ppm as singlets in a ratio of $\text{H}_a:\text{H}_b = 1:1$, which

further confirmed the structure of methacrylated VK20. The methylene protons at 3.15 ppm nearby the amine groups of lysine residues ($-\text{CH}_2\text{-NH}_2$) were assigned as H_c and the methyl protons of the valine residue ($-(\text{CH}_3)\text{-CH}_3$) at 0.94 ppm as H_d . The ratio of the integrals $\text{H}_a:\text{H}_b:\text{H}_c:\text{H}_d = 1.05:1.05:16:55$ matches with the theoretical ratio of 1:1:16:54. The proton NMR spectrum of monomer VK20 solution suggested a random and fast reorientation of the conformation of the peptide molecules. When the pH of peptide solution was adjusted to 9.2 by addition of NaOD, gelation occurred. In contrast, the broad spectrum (Figure 4b) indicates an arrangement of the peptides into a fibrillar network. Specifically, the signals of protons almost disappeared as a result of the β -sheet aggregation of VK20. These results further confirmed the sol-gel transition of VK20 into a physical gel network.

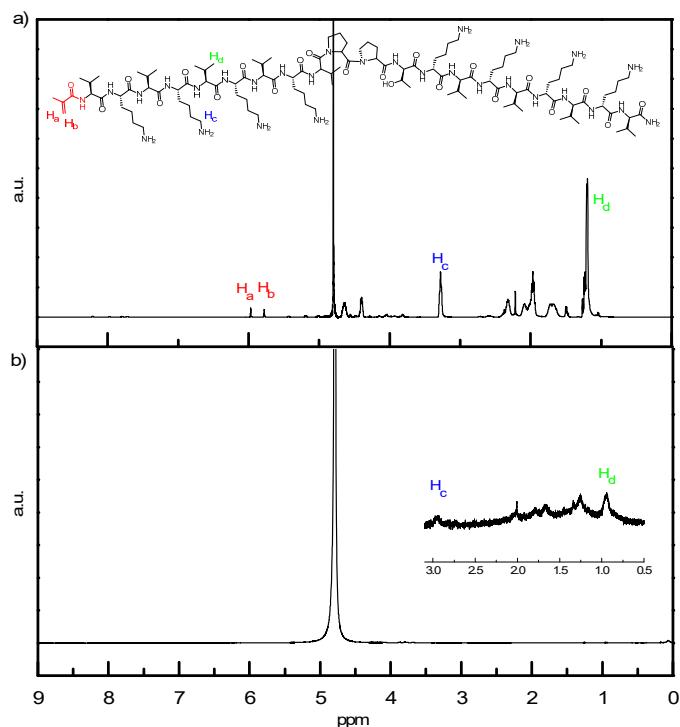


Figure 4 ^1H NMR spectroscopic characterization of VK20 peptide solutions: a) 1 wt% VK20 D_2O solution, pH 4.5; b) after addition of concentrated NaOD, where a gelation was observed (pH 9.2).

The nanostructure of the physically crosslinked VK20 gel was studied by TEM. Uranyl acetate was used as a negative stain for the self-assembled fibrils to enhance the contrast. At pH 9, VK20 (with 1 wt%) formed a hydrogel network. In the TEM image (Figure 5) of this hydrogel a highly interconnected fibrillar network structure at the nanoscale was observed, attributed to

self-assembly. VK20 consists of two β -strands of alternating valines and lysines, which are connected by a β -turn. In the self-assembly process, the amphiphilic β -sheet structure forms bilayer-structured fibrils composed of a hydrophobic valine core, which assembles facially through hydrophobic association and laterally via intermolecular hydrogen bonding [19]. The crosslinks formed between the fibrils can be attributed to the entanglement of self-assembled fibrils or defect-induced branching [20]. Thus, the width of fibril defined the length of VK20. The theoretical length of the unfolded VK20 was estimated to be 7.0 nm. The formed fibrils showed a uniform width of 3.4 ± 0.5 nm (N=30). This size fits well with the theoretical value of the folded β -hairpin peptides (i.e. strand length), which is slightly larger than MAX1 (3.2 nm) due to the additional methacrylate group. These results supported the potential mechanism of self-assembly, indicating that VK20 folds intramolecularly into a β -hairpin rather than into an extended β -sheet.

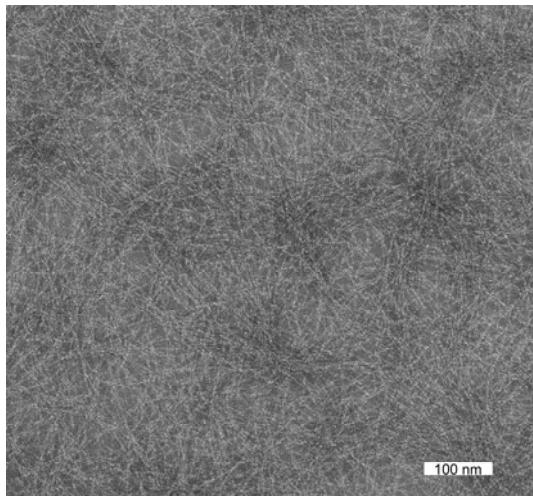


Figure 5 TEM image of 0.05 wt% VK20 gel (diluted from 1 wt%) at pH 9.

CONCLUSIONS

The pH-responsive conformational transition of a peptide that was functionalized with methacrylate at the N-terminus was investigated. VK20 folded into a β -hairpin structure when the pH was raised above 9. This transition was attributed to the lowered electrostatic repulsion between protonated lysine groups, which consequently increased intramolecular hydrophobic interactions and hydrogen bonding. The folded peptides assembled together resulting in a physically crosslinked hydrogel network with well-defined thickness caused by the facial

hydrophobic interactions and lateral intermolecular hydrogen bonds. Compared to the non-methacrylated peptide MAX1 the N-terminal methacrylated peptide showed a faster conformational change, which can be attributed to the decreased net charge and the increased hydrophobicity of VK20. The kinetics of conformational change of VK20 between random coil and β -hairpin can be tuned by adjustment of the pH ranging between 5 and 9.5. Furthermore, the rates of β -sheet assembly and the mechanical responsiveness can be tailored by varying the pH from 8.5 to 9.5. This study showed that the modification of the peptide MAX1 with a methacrylate group does not alter its ability for conformational changes in general. Thus, VK20 is a promising reactive peptide precursor for further studies, such as conjugation or polymerization.

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