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Self-Assembled Hydrogels from Poly[*N*-(2-hydroxypropyl) methacrylamide] Grafted with β -Sheet Peptides

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Abstract

A new hybrid hydrogel based on poly[*N*-(2-hydroxypropyl)methacrylamide] grafted with a β -sheet peptide, Beta11, was designed. Circular dichroism spectroscopy indicated that the folding ability of β -sheet peptide was retained in the hybrid system, whereas the sensitivity of the peptide towards temperature and pH variations was hindered. The polymer backbone also prevented the twisting of the fibrils that resulted from the antiparallel arrangement of the β -strands, as proved by Fourier transform infrared spectroscopy. Thioflavin T binding experiments and transmission electron microscopy showed fibril formation with minimal lateral aggregation. As a consequence, the graft copolymer self-assembled into a hydrogel in aqueous environment. This process was mediated by association of β -sheet domains. Scanning electron microscopy revealed a particular morphology of the network, characterized by long-range order and uniformly aligned lamellae. Microrheology results confirmed that concentration-dependent gelation occurred.

Keywords

hybrid hydrogels; β -sheet fibrils; poly[*N*-(2-hydroxypropyl)methacrylamide] [poly(HPMA)]; graft copolymers; self-assembly

Introduction

The self-assembly of peptides and proteins into hierarchical β -sheet structures has attracted considerable research activity. In particular, polymer scientists have used peptides to achieve precise control over the architecture and nanostructure of synthetic polymers by mediating their organization. Numerous reports have shown that the intrinsic self-assembly ability is retained in hybrid block copolymers obtained by conjugation of various β -sheet peptides with poly(ethylene oxide) (PEO),^{1–3} poly(ethylene glycol) (PEG),^{4–7} as well as poly(*N*-butyl acrylate)⁸ and poly[*N*-(2-hydroxypropyl)methacrylamide] [poly(HPMA)].⁹ Moreover, our laboratory designed and synthesized hybrid hydrogels whose self-assembly was mediated by

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coiled-coil motifs, non-covalently attached¹⁰ or covalently grafted¹¹ to a poly(HPMA) backbone. However, research on structure regulation in β -sheet peptides-grafted copolymers has been scarce so far. Few available reports on polyglutamate-grafted polyallylamine¹² and poly(L-leucine) grafted polyallylamine¹³ copolymers showed that the formation of amyloid-like fibril structure could be easily controlled by manipulating the pH towards acidic conditions. In addition, introduction of a β -sheet forming Gly-Ala-Gly-Ala tetrapeptide as grafts onto polyferrocenylsilane resulted in β -sheet structural element formation in the metallopolymer-peptide conjugate.¹⁴ To our knowledge, the possibility to create hybrid hydrogels by using β -sheet peptide grafts as physical crosslinkers was not yet exploited. Previously obtained hybrid hydrogel systems based on β -sheet motif either used the immunoglobulin (Ig)-like domain muscle protein, titin, to crosslink acrylamide (AAM) copolymers via metal coordination,¹⁵ or the “click” reaction to synthesize a PEO-tetraphenylalanine conjugate that self-assembled into nanotubes, entangled at higher concentrations into soft gels.¹⁶

Encouraged by previous results,⁹ we anticipated that the conjugation of a β -sheet peptide as grafts on poly(HPMA) would result in the formation of a fibril-like nanostructure for the poly(HPMA)- β -sheet copolymer, and with increasing concentration, of a hybrid hydrogel. Preserving the β -sheet arrangement in a graft copolymer might be a challenging task considering that in order to self-assemble, two β -strands should be no further away from each other than 4.7 Å,¹⁷ a condition that might be prevented by the random nature of the graft distribution on the polymer backbone. In constructing β -sheet graft copolymers of poly(HPMA) we employed a simple strategy that although did not resolve the problems associated with the random distribution of the grafts, it allowed the retention of the β -sheet structure. A short peptide, Beta11, possessing a high tendency of β -sheet formation and, with increasing concentration, to self-associate into gels, was designed following Aggeli's model.¹⁸ The peptide was synthesized using solid-phase methods and manual 9-fluorenyl-methoxycarbonyl (Fmoc)/tert-butyl (tBu) strategy. Furthermore, Beta11 sequence was modified at its *N*-terminus with a short Cys-Gly-Gly tripeptide spacer intended to decrease the steric hindrance of the polymer backbone on the formation of β -sheets and to provide an attachment point. The graft copolymer was finally prepared via a maleimide-thiol coupling reaction between the maleimido-modified poly(HPMA) side chains and the cysteine-terminated Beta11 peptide. By taking advantage of the poly(HPMA) random coil conformation in water,¹⁹ the Beta11 grafts attached via thioether bonds were expected to self-assemble, thus imposing the peptide strong tendency to associate into β -sheet fibrils, and to form gels, onto the copolymer. The β -sheet secondary structure of the peptide, and copolymer was characterized by circular dichroism (CD), Fourier transform infrared (FTIR) spectroscopy, thioflavin T (ThT) binding studies, and transmission electron microscopy (TEM). The morphology of the self-assembled, physical gel networks was investigated by scanning electron microscopy (SEM). To gain insight into the mechanism of gelation, gel formation with respect to concentration was studied by microrheology.

Experimental Part

Materials

Fmoc-protected amino acids and rink amide 4-methyl-benzhydrylamine (MBHA) resin were purchased from Novabiochem (San Diego, CA). 2-(7-Aza-1*H*-benzotriazole-1-yl)-1,1,3,3-tetramethyl-uronium hexafluorophosphate (HATU, >98%) and 1-hydroxybenzotriazole (HOBt) were from AK Scientific (Mountain View, CA). *N,N*-dimethylformamide (DMF, 99.8%), *N,N*-diisopropylethylamine (DIPEA, 99%), piperidine (99.5+%, Biotech grade), Triton X-100, acetic anhydride (99+%), triisopropylsilane (TIS, 99%), triethylamine (99%), methanol, and thioflavin T (ThT), and thioflavin S (ThS) dyes were from Sigma-Aldrich (St. Louis, MO). *N,N*-diisopropylcarbodiimide (DIC, >98%), tris(2-carboxyethyl)phosphine (TCEP) and ethanedithiol (EDT, >98%) were from Fluka (Milwaukee, WI). Lithium bromide

(LiBr, anhydrous, 99+%) was purchased from Strem Chemicals (Newburyport, MA). Congo red (CR) dye was from Alfa Aesar (Ward Hill, MA). Trifluoroacetic acid (TFA, 99%) and 1-methyl-2-pyrrolidinone (NMP) were purchased from Acros Organics (Morris Plains, NJ). Diethyl ether and dichloromethane (DCM) were from Maleinckrodt Baker (Philipsburg, NJ). 2,2'-Azobisisobutyronitrile (AIBN) and succinimidyl *trans*-4-(maleimidylmethyl)cyclohexane-1-carboxylate (SMCC) were from Soltech Ventures (Beverly, MA). Deuterium oxide (D₂O, 99.9%) was from Cambridge Isotope Laboratories (Andover, MA). *N*-(3-aminopropyl)methacrylamide (MA-NH₂) was purchased from Polysciences (Warrington, PA), and *N*-(2-hydroxypropyl)methacrylamide (HPMA) was synthesized as previously described.²⁰

β-Sheet Peptide Synthesis

Beta11, Ac-Thr-Thr-Arg-Phe-Thr-Trp-Thr-Phe-Thr-Thr-Thr-amide, was synthesized using solid-phase methodology and manual Fmoc/tBu strategy on rink amide MBHA resin following a protocol similar to one previously described.²¹ After swelling of the resin beads (200 mg, 0.11 mmol) in DCM (5 mL), and deprotection with 20% piperidine in DMF (2.5 mL), the first amino acid, Fmoc-Thr(tBu)-OH, dissolved in DMF, was attached to the resin in the presence of HOBt and DIC. The rest of the amino acids (each 0.348 mmol) were dissolved in DMF/HOBt and attached to the resin beads after deprotection, one at a time, in the presence of HATU and DIPEA (each 0.348 mmol). During synthesis, aggregation was overcome by washing the resin with a solution of 0.8 M LiBr in DMF before the coupling of Fmoc-Trp(Boc)-OH, and by using 20% piperidine in DCM/DMF/NMP (1:1:1) mixture with 1% Triton X-100 for Fmoc-cleavage. The completion of each coupling step was verified by Kaiser test. After coupling 11 amino acid residues, part of the resin-bound peptide was treated with a solution of acetic anhydride and DIPEA in DCM for acetylation of *N*-terminus, and then with TFA/TIS/H₂O (95:2.5:2.5 vol.-%) cocktail solution for cleavage of the peptide from the resin beads and deprotection of the side chains. The product (Beta11) was used for control experiments. The main fraction of the resin-bound peptide was modified by the coupling of three amino acid residues, Cys-Gly-Gly, resulting in resin-bound peptide CGGBeta11; the latter was cleaved with TFA/H₂O/EDT/TIS (94:2.5:2.5:1 vol.-%) solution. Crude peptides were precipitated with cold diethyl ether, collected by filtration, dried in air, dissolved in de-ionized (DI) water and lyophilized. Beta11 and CGGBeta11 identities were confirmed by MALDI-TOF mass spectrometry (MS; Voyager-DE STR Biospectrometry Workstation, PerSeptive Biosystems, Framingham, MA), showing a single peak corresponding to the expected molecular weight, at $M^+ = 1403.96$ m/z (theoretical 1403.67 m/z) for Beta11, and $M^+ + 1 = 1579.77$ m/z (theoretical 1577.73 m/z) for CGGBeta11. Purity analysis (>80%) by analytical reversed phase (RP)-HPLC (Agilent Technologies), equipped with Eclipse XDB-C8 column (4.6 × 250 mm, 5 μm particle size, 300 Å pore size) and detectors at 220 and 280 nm, revealed that the peptides could be used without further purification.

Synthesis and Purification of Poly(HPMA) Grafted with β-Sheet Peptide [Poly(HPMA)-g-CGGBeta11]

Poly(HPMA)-g-CGGBeta11 was synthesized following a procedure similar to the one described previously for HPMA graft copolymers containing coiled-coil domains.¹¹ First, radical copolymerization in methanol at room temperature, using AIBN as the initiator was employed to obtain a copolymer of HPMA and *N*-(3-aminopropyl)methacrylamide (MA-NH₂). The number-average molecular weight and molecular weight distribution of the copolymer were measured by size exclusion chromatography (SEC) on an ÄKTA FPLC system (Amersham Pharmacia Biotech) equipped with UV and RI detectors, using a Superose 6 column, previously calibrated with poly(HPMA) fractions, and phosphate buffer solution (PBS, pH = 7.2) as eluent. The estimated number-average number molecular weight and polydispersity were $M_n = 4.3 \times 10^4$ g·mol⁻¹, and $M_w/M_n = 1.92$, respectively. In the second step,

the linear HPMA copolymer with pendant amino groups was reacted with heterobifunctional reagent SMCC in DMF, in the presence of triethylamine, to provide a HPMA copolymer with side chains functionalized with maleimide groups. The content of maleimide groups was $4.95 \times 10^{-7} \text{ mol} \cdot \text{mg}^{-1}$ (on average, about 21 maleimide groups per macromolecule) as determined by modified Ellman assay. Ninhydrin test showed that the content of free, pendant amino groups on the HPMA copolymer chain was negligible after modification with SMCC. Finally, the cysteine modified β -sheet peptide, CGGBeta11, pre-incubated with TCEP, was attached to the HPMA copolymer side chains via thioether bonds. A ratio of maleimide:cysteine = 2:1 was used when mixing the reactants in DMF/DI water solution. Typically, a solution (8 mg/500 μL) of Beta11 in DMF was mixed with an HPMA aqueous solution (8 mg·mL⁻¹). The thiol-maleimide coupling reaction took place in 24 h at room temperature and was monitored by analytical RP-HPLC. The product was purified by dialysis (using a membrane with molecular weight cut-off, MWCO, 6000–8000 Da) against water 48 h to remove any unreacted CGGBeta11 peptide. The peptide content in the copolymer was estimated to be approximately 24 wt % (or an average of 5.5 peptide grafts per polymer chain) by UV-Vis spectroscopy, based on Trp absorption (molar extinction coefficient at 278 nm, $\epsilon = 5579 \text{ M}^{-1} \cdot \text{cm}^{-1}$). Considering the maleimide:cysteine ratio used, the yield of the coupling reaction was 52%.

Circular Dichroism Spectroscopy

CD spectra were collected on an Aviv 410 CD spectrometer (Biomedical Inc., Lakewood, NJ) equipped with a thermoelectric temperature-control system. Samples were prepared by dissolving a known mass of lyophilized product in DI water. When needed, DI water at pH = 2 or 11 adjusted by addition of appropriate amounts of 1 N HCl or 1 N NaOH solutions, respectively, as well as PBS solution at pH = 7 were used. Solutions (with peptide concentrations determined by UV spectroscopy) were incubated at room temperature for 3 d prior to CD measurements. Wavelength scans were recorded at 20 °C, from 250 to 195 nm, at 1 nm intervals and 3 s averaging time, using a 0.1 cm path length quartz cuvette. Spectra were averaged from three consecutive scans and were background corrected. Ellipticity was reported as mean residue ellipticity ($[\theta]$, deg·cm²·dmol⁻¹), calculated as previously described.²² The thermal stability of Beta11 peptide and of poly(HPMA)-g-CGGBeta11 copolymer was studied by monitoring the change of ellipticity at 218 nm as a function of temperature between 4 and 91 °C, using a temperature step of 3 °C, and an equilibration time of 3 min for each step. Two heating and cooling cycles were recorded.

Fourier Transform Infrared Spectroscopy

FTIR investigation of Beta11 and poly(HPMA)-g-CGGBeta11 secondary structure was carried on a Biorad FTS-6000 spectrometer (Cambridge, MA) equipped with a liquid nitrogen cooled MCT detector. Liquid FTIR samples (3 mg·mL⁻¹ in D₂O) were prepared for both peptide and copolymer, incubated for a few days at room temperature, and checked by CD prior to measurements. A transmittance liquid cell with CaF₂ windows and 50 μm spacers were used. FTIR spectra were recorded at a resolution of 4 cm⁻¹, averaged from 500 consecutive scans, and background corrected. Second-derivative calculations for each spectrum were performed for an easier identification of the characteristic peaks positions.

Thioflavin T Binding Studies

ThT fluorescence measurements were conducted on a LS-55 luminescence spectrometer (Perkin-Elmer), using a 1 cm path length quartz cuvette. Emission wavelength scans were recorded between 540 nm and 460 nm, with an excitation wavelength of 440 nm, at 1 nm sampling interval, and a scan speed of 100 nm·min⁻¹. Spectra were averaged from 10 consecutive scans. A 100 μM stock solution of ThT in DI water, and solutions of Beta11 and poly(HPMA)-g-CGGBeta11 (1 mg·mL⁻¹) with β -sheet structure were used for samples

preparation. Concentrations of the peptide and of the ThT dye in the final samples were around 50 μM , well above the ThT critical micellar concentration.²³ Measurements were taken immediately following sample preparation. For comparison purposes, a similar protocol was employed when preparing samples for ThS fluorescence measurements, whereas CR binding experiments were performed as previously described.⁹

Transmission Electron Microscopy

TEM micrographs were obtained on a Philips Tecnai transmission electron microscope operated at 100 kV accelerating voltage and magnifications ranging from 3900 to 9700 times. Samples were prepared on copper specimen grids coated with carbon support film (CF200-Cu; Electron Microscopy Sciences, Fort Washington, PA). Dilute aqueous solutions of Beta11 and poly(HPMA)-*g*-CGGBeta11 (0.5 $\text{mg}\cdot\text{mL}^{-1}$) having a β -sheet secondary structure as confirmed by CD were used for sample preparation. Each grid was placed with the carbon-coated face on top of a 20 μL drop of corresponding solution for 20 s, and then negatively stained with uranyl acetate solution 4% (w/v) for another 20 s. Excess solution was drained off the grids. The stained samples were left to dry overnight and then examined.

Hydrogel Preparation

Gels were obtained by dissolving known amounts of lyophilized Beta11 peptide or poly(HPMA)-*g*-CGGBeta11 copolymer in DI water. Typically, peptide samples having a concentration of 0.8 wt %, and copolymer samples at 3 wt % formed gels within minutes, whereas lower concentration solutions gelled after incubation for several days at room temperature.

Scanning Electron Microscopy

Hydrogel samples of Beta11 and poly(HPMA)-*g*-CGGBeta11, prepared as described above, were shock-frozen by immersion in liquid nitrogen for 10 min, and then lyophilized overnight. Freeze-dried samples were mounted on SEM aluminum stubs and sputtered with gold for 20 s to obtain a conductive coating visible by SEM. The observation of the hydrogels morphology at the surface, as well as in bulk, was carried on a scanning electron microscope (Hitachi S-2460N SEM) at an accelerating voltage of 20 kV and magnifications from 70 to 600 times.

Microrheology

Microrheological measurements were performed as previously described.²⁴ Surfactant free fluorescent yellow-green amidine modified polystyrene (PS) beads (Interfacial Dynamics Corp., Portland, OR) with 0.52 μm in diameter were dispersed into the peptide/copolymer solutions having concentrations ranging from 0.1 to 3 wt %. The samples were immediately sealed between a No. 1.5 glass slide and a glass coverslip with melted parafilm and then visualized on a Nikon Eclipse E800 microscope equipped with a 100 $\times$ oil-immersion objective. The samples were focused in a manner that avoided wall effects. The Brownian motion of the PS beads for each sample was analyzed by IDL software (Research Systems Inc., Boulder, CO) based on 3000 consecutive images captured by a CCD camera (Dage-MTI, DC330) and recorded at intervals of 33 ms using StreamPix software (Norpix, Montreal, Canada). The mean square displacement (MSD) of tracer particles, the storage modulus, $G'(\omega)$, and the loss modulus, $G''(\omega)$, of the gels were determined using algorithms previously developed.²⁵

Results and Discussion

β -Sheet Peptide Design

Beta11 is an undecapeptide whose sequence was designed based on P11-3 peptide described by Aggeli et al.,¹⁸ with replacement of glutamine (Gln) with threonine (Thr), as shown in Table

1. Thr is a polar residue with high propensity for β -sheets, and it was chosen to enhance the solubility of Beta11. Phenylalanine (Phe) was kept in positions 4 and 8, and tryptophan (Trp) in position 6, due to proven ability to promote the hierarchical self-assembly of the β -sheets into fibrils via hydrophobic interactions.¹⁸ In addition, arginine (Arg) in position 3 was also an useful design criterion which favored the formation of antiparallel β -sheet structure in DI water and permitted peptide self-assembly over a wide pH range. To create an attachment point onto the poly(HPMA) backbone and to decrease the steric hindrance that the polymer might cause during the self-assembling process, the *N*-terminus of Beta11 was further modified with a Cys-Gly-Gly tripeptide spacer. Both peptides, Beta11 and CGGBeta11, were obtained through manual solid-phase synthesis using the Fmoc/tBu strategy. CD spectroscopy demonstrated that CGGBeta11 had similar folding ability to Beta11. The former was used for conjugation to poly(HPMA) polymer chains, whereas the latter was used as a control.

Synthesis and Characterization of Poly(HPMA) Grafted with β -Sheet Peptide

The synthesis of the poly(HPMA)-*g*- β -sheet graft copolymer was performed as illustrated in Figure 1. By taking advantage of the flexibility of the HPMA polymer backbone,¹⁹ the CGGBeta11 grafts attached via thioether bonds were expected to self-assemble, thus preserving the strong tendency of the peptide to associate into β -sheets and to form gels. The density of the grafts, the concentration, and the incubation time were factors essential for the self-assembly.

The secondary structure of poly(HPMA)-*g*-CGGBeta11 was evaluated by CD spectroscopy and compared with that of Beta11 alone. Solutions of the graft copolymer or Beta 11 peptide in DI water, with concentrations between 0.5 and 4 mg·mL⁻¹ and incubated few days at room temperature, showed a characteristic β -sheet CD profile with a minimum at 218 nm. Temperature dependent CD measurements were employed to investigate the β -sheet structure stability. Solutions of Beta11 peptide and of poly(HPMA)-*g*-CGGBeta11 copolymer in water (1 mg·mL⁻¹) were examined. On increasing the temperature to 91 °C, the CD spectrum of Beta11 changed to a minimum around 200 nm, typical for a random coil structure of the peptide⁷ (Figure 2, A, C). The Beta11 melting point, the temperature at which the peptide becomes unfolded, was estimated as 50 °C. Upon cooling, the folding was not reversible under experimental conditions used. The β -sheet structure of the peptide only recovered after incubation at room temperature for several days. On the contrary, the CD spectra of poly(HPMA)-*g*-CGGBeta11 copolymer did not change on increasing the temperature from 4 to 91 °C (Figure 2, B, C), nor on subsequent cooling. The fact that the copolymer self-assembly did not depend on temperature can be explained by the stabilizing influence of the poly(HPMA) polymer backbone on the secondary structure of the peptide, previously observed for block²⁶ and graft¹¹ copolymers with coiled-coil domains, and block copolymers with β -sheet domains.²⁷ It seems that the β -sheet structure of the peptide grafts was “locked-in” by the polymer backbone and was not influenced by the fluctuations in temperature. Moreover, the shielding effect of the polymer on the peptide conformation was evidenced during pH-dependent experiments, performed at 20 °C. Beta11 peptide possessed a β -sheet secondary structure at acidic pH, but was largely unordered at pH 7 and 11 (Figure 2, D). However, upon conjugation to poly(HPMA), the pH sensitivity of the peptide secondary structure in graft copolymer was significantly reduced and the β -sheets were stabilized (Figure 2, E). Only a small decrease in the intensity of the minimum at 218 nm was found at pH 11, probably due to the deprotonation of Arg side chains. When explaining the insensitivity of the hybrid conjugate to temperature and pH changes, the steric hindrance effect from the polymer backbone should also be considered.

FTIR of aqueous solutions confirmed the CD results. Predominant peaks were detected for Beta11 peptide at 1618 cm⁻¹ (Figure 3, A) and for poly(HPMA)-*g*-CGGBeta11 graft

copolymer at 1617 cm^{-1} (Figure 3, B); these peaks are in the $1614\text{--}1620\text{ cm}^{-1}$ range, indicating a high β -sheet content and aggregation.⁶ Additional peaks at 1672 cm^{-1} with a shoulder at 1692 cm^{-1} for the Beta11 peptide, and at 1673 cm^{-1} with a shoulder at 1693 cm^{-1} for the graft copolymer were assigned to antiparallel β -sheets, taking into account that peaks characteristic to such an arrangement are shifted to lower wavenumbers when the medium is D_2O .²⁸ The FTIR spectra also revealed an intense band attributed to helical conformation in Beta11 (at 1652 cm^{-1}), whereas in the graft copolymer, the analog band was considerably weaker than the β -sheet bands. This behavior was probably caused by the fact that in poly(HPMA)-g-CGGBeta11 twisting of the β -sheets to self-assemble into fibrils was prevented by the steric hindrance imposed by the polymer backbone. No evidence for the presence of parallel β -sheets, which would have given rise to a peak at approximately 1645 cm^{-1} ,⁴ was found.

Formation of β -sheet fibrils in both peptide and HPMA graft copolymer was further indicated by the results of ThT binding experiments. ThT is a fluorescent benzothiazole dye with high selectivity for amyloid-like fibrils to which it binds parallel to their long axis. When bound to β -sheet fibrils, ThT showed an increase in fluorescence emission (λ_{em}) at around 485 nm when an excitation wavelength (λ_{exc}) of 440 nm has been used.^{29,30} We chose this dye due to its small tip-to-tail length ($\sim 15\text{ \AA}$), that promotes fibril-like affinity even with only four or five associated β -strands,³⁰ which can be advantageous when studying the poly(HPMA)-g-CGGBeta11 fibrils. On the contrary, larger dye molecules, like CR and ThS, require a higher number of self-assembled β -strands, therefore binding studies using these two dyes were performed only for comparison. Fluorescence emission spectra ($\lambda_{\text{exc}} = 440\text{ nm}$) of Beta11 and its conjugate, poly(HPMA)-g-CGGBeta11 in the presence of $50\text{ }\mu\text{M}$ ThT showed that upon binding to the fibrils, there was an enhancement of the fluorescence at 488 nm compared to the blank, ranging from around 1.5-fold, in the peptide case, to around 3.5-fold, in the graft copolymer case (Figure 4, A). At similar peptide concentrations, the poly(HPMA)-g-CGGBeta11 displayed higher ThT fluorescence enhancement than Beta11. Binding to the Beta11 fibrils might be prevented to a certain extent by the electrostatic repulsion mediated by protonated Arg side chains and the positively charged ThT molecule. In contrast, the shielding effect provided by the polymer backbone, as well as the ThT molecule length, are probably factors that favor a better binding of ThT to the graft copolymer fibrils. Indeed, comparative studies showed that for larger negatively charged molecules of CR and ThS, a better binding, probably via electrostatic interactions, was obtained in the case of the peptide alone, whereas the size of the dyes greatly affected binding to the copolymer. For example, CR binding to the β -sheets of Beta11 peptide resulted in a shift of the CR characteristic intense peak from 498 nm to 508 nm, whereas binding of CR to the copolymer produced a negligible shift to 501 nm (Figure 4, B). The point of maximal spectral difference (λ_{max}) at 527 nm further confirmed the existence of the β -sheets in the peptide,⁹ however the value obtained for the poly(HPMA)-g-CGGBeta11 copolymer ($\lambda_{\text{max}} = 501\text{ nm}$) supported the hypothesis according to which the size of the dye prevented the CR binding from occurring (Figure 4, C).

β -Sheet fibril formation, proved by ThT binding studies, was also confirmed by TEM imaging. Examination of samples dried from aqueous solutions showed the existence of micrometer-long fibrils in both, Beta11 peptide and poly(HPMA)-g-CGGBeta11 graft copolymer. The fibrils were few tens of nm in width, characteristic of a typical amyloid morphology, although the exact estimation of the individual fibril width was difficult due to lateral aggregation. Interestingly, while the peptide fibrils were strongly associated into bundles up to $3\text{ }\mu\text{m}$ wide (Figure 5, A), the copolymer fibrils showed little lateral aggregation (Figure 5, B). Moreover, most of the copolymer fibrils were aligned, running parallel to each other and maintaining an even spacing in between them. This phenomenon was previously observed by Collier et al. for β -sheet-PEG block copolymers,⁶ and is an indication of an alternating layered packing of the poly(HPMA)-g-CGGBeta11 copolymer fibrils. Since formation of spherical or petal-like micelles, that may result from intramolecular interactions between grafts, was not observed in

TEM imaging, these results, together with those obtained from FTIR experiments, support our hypothesis that self-assembly of the copolymer takes place through favorable intermolecular association of the grafts into antiparallel β -sheets.

Characterization of Hydrogels Self-Assembled by β -Sheet Peptide Domains

Beta11 peptide and poly(HPMA)-g-CGGBeta11 graft copolymer were soluble in water. Conjugation of Beta11 with the hydrophilic poly(HPMA) significantly improved its solubility. Peptide solutions having concentration of 0.8 wt %, and copolymer solutions with concentration of 3 wt % formed gels within minutes, whereas lower concentrations gelled in time, only after incubation for several days at room temperature. Applying mechanical forces, like vortex or agitation, easily caused disassembly of the metastable structure and dissolution of the networks. The gels could then reassemble on standing at room temperature, indicating their thixotropic nature. While the effects of environmental conditions, such as concentration, time, pH, and mechanical factors were important, in case of the copolymer, the density of the β -sheet grafts was influential on the gelation process. It seems that in order to reach the gel point in the hybrid copolymer system, a certain number of physical crosslinking points, or self-assembled grafts, on the polymer backbone was needed. When a maleimido-modified HPMA copolymer having a concentration of 3.56×10^{-7} mol·mg⁻¹ maleimide groups (15 per chain) was used for the synthesis of the poly(HPMA)-g-CGGBeta11 graft copolymer following the protocol described above, a gel could not be obtained, presumably due to the lower density of the resulting β -sheet grafts on the polymer backbone. This kind of dependency was previously observed in our laboratory for hybrid hydrogels self-assembled from HPMA copolymers containing coiled-coil grafts.²²

Morphology of the hydrogels was investigated by SEM, which showed highly porous structures for both peptide and copolymer gel (Figure 5, C, D). The regular distribution of the polygonal pores provided a leaf-like appearance on the gels surface, indicating that a large amount of water was retained by the physically crosslinked networks. The pores of the Beta11 peptide gel were estimated as being about 4 times larger than those of poly(HPMA)-g-CGGBeta11 copolymer gel. Interestingly, the SEM micrograph of the fractured central region of the copolymer gel revealed a microscopic aligned lamellar architecture formed of closely packed fibrils (Figure 5, D inset). The lamellae were separated by thin partitions, probably corresponding to the poly(HPMA) backbone, and their orientation was perpendicular to the hydrogel surface plane. Similar lamellar structures have been observed for P11-1 and P11-2 peptides,^{31,32} gelatin,³³ chitosan,³⁴ as well as for poly(L-lactic acid) (PLLA) and poly(D,L-lactic acid-co-glycolic acid) (PLGA85/15),³⁵ and were considered the result of the freeze-drying process. It was hypothesized that during freezing, the ice crystals push the fibrils in the hydrogel, therefore causing their alignment with respect to each other and perpendicular to the hydrogel surface plane, based on physical rather than chemical interactions. However, the lamellar morphology observed for the poly(HPMA)-g-CGGBeta11 freeze-dried gel was highly organized when compared to previous reports on similar networks.^{31–34} The long-range order of the aligned domains having widths and lengths of several hundreds of micrometers, and the uniformity of the lamellae was not seen in any of the previous studies mentioned above. One factor, which might contribute to this difference in architecture, is the phase separation between the peptide and the polymer that possibly influenced the crystallization of the system. More intriguingly, the lamellar structure of the freeze-dried HPMA graft copolymer gel resembled the organization of copolymer fibrils revealed by TEM imaging (Figure 5, B). This result suggests that, unlike previously described systems, the lamellar architecture of poly(HPMA)-g-CGGBeta11 copolymer is not only the consequence of the freeze-drying process, but rather of a combination of factors among which the self-assembly of the fibrils inside the gel plays the major role.

The gelation of Beta11 peptide and poly(HPMA)-*g*-CGGBeta11 graft copolymer was further evaluated by microrheology measurements. The double logarithmic plots of the mean-square displacement (MSD) of tracer particles versus lag time constructed for each system at different concentrations showed linear dependence with slopes between 0 and 1 for low concentration samples. On the contrary, at higher concentrations of 0.8 wt % Beta11 and 3 wt % poly(HPMA)-*g*-CGGBeta11, no dependence in the MSD (slope 0) was observed (Figure 6, A, B). The linear increase of MSDs with time was an indication that the particles diffused freely in a liquid environment. Once the Brownian motion of the tracer particles was heavily restricted in a gel-like environment, the MSD reached a plateau over time. Beta11 was predominantly viscous at concentrations of 0.1–0.3 wt %, viscoelastic at 0.4 wt %, and purely elastic at 0.8 wt % (Figure 6, A). Poly(HPMA)-*g*-CGGBeta11 required higher concentrations to induce viscoelasticity. Up to around 2 wt %, the graft copolymer displayed a viscous behavior, which became slightly viscoelastic around 2.5 wt %, and finally, purely elastic at 3 wt % (Figure 6, B). This result confirmed the formation of the hybrid hydrogel, and suggested that the gelation process was dependent not only on the incubation time, but also on the concentration of the β -sheet domains in the system. The gelation of the peptide and of the copolymer, respectively, was also characterized by measuring the evolution of storage modulus, G' (elastic component of the stress), and of loss modulus, G'' (viscous component of the stress), as a function of frequency (Figure 7, A, B). The gel behavior at 0.8 wt % peptide, and 3 wt % copolymer was clearly depicted by higher G' values when compared to G'' values. In fact, for predominantly viscous solutions, such as Beta11 below 0.4 wt %, and poly(HPMA)-*g*-CGGBeta11 below 2.5 wt %, G'' significantly exceeded G' , therefore G' could not be calculated being 100 times smaller. In contrast, with increasing concentration, for predominantly elastic solutions at 0.8 wt % peptide and 3 wt % copolymer, G' became 2 degrees of magnitude larger than G'' . G' was insensitive to frequency and showed a plateau-like behavior, another indicator for gel formation in both systems. Critical concentrations separating a liquid-like ($G'' > G'$) and a gel-like ($G' > G''$) response were at about 0.4 wt % peptide and about 2.5 wt % graft copolymer. The viscoelastic character of the critical concentrations was confirmed by the similar magnitude of G' and G'' , calculated and represented in the frequency sweep plots for solutions of Beta11 peptide and poly(HPMA)-*g*-CGGBeta11 graft copolymer (Figure 7, A, B). While a detailed study on viscoelastic properties of these systems would require a comparison of the results obtained from microrheology with those obtained from conventional rheometry, previous reports on similar fibrillar gels³⁶ showed that, in the frequency range tested herein, there is a good agreement between measurements.

Conclusions

A novel hybrid hydrogel system based on a HPMA copolymer grafted with a β -sheet forming peptide was designed and evaluated. For its synthesis, a short β -sheet forming peptide, CGGBeta11, was coupled, via thioether bonds, to maleimido-modified side chains of HPMA copolymer. CD, FTIR and ThT binding experiments indicated that the strong tendency of the peptide to self-assemble into β -sheets was retained after conjugation with the polymer. The polymer had a shielding effect on the peptide grafts, decreasing their sensitivity to temperature and pH variations, and favoring the preferential binding of the fluorescent ThT dye. The polymer backbone was also responsible for hindering the twisting of the fibrils formed through the antiparallel arrangement of the peptide β -strands. TEM imaging showed that the graft copolymer formed dramatically different fibrils from the peptide; while Beta11 fibrils were characterized by pronounced aggregation, the poly(HPMA)-*g*-CGGBeta11 fibrils formed ordered matrices in which lateral aggregation was minimal. Consequently, the tendency of the peptide to form hydrogels was preserved in the copolymer, depending on the density of the grafts onto the polymer backbone, on the concentration, and on the incubation time. Gels were formed in the peptide at concentrations as low as 0.8 wt %, whereas higher concentrations, 3 wt %, were needed for the copolymer. Investigation of network morphology by SEM revealed

that the copolymer hydrogel was characterized by the existence of a long-range order with uniformly aligned lamellae, a result of the self-assembly process. The confirmation of hydrogel formation was obtained through microrheology measurements, which proved that at the gel point, the systems displayed purely elastic behavior, depicted by plateau formation in MDSs, and higher G' values when compared to G'' . Along with previous work,^{9–11} this study demonstrated that secondary structures can be imposed on hybrid HPMA copolymers grafted with β -sheet forming peptides and that hybrid hydrogels with particular properties can be obtained by self-assembly. Such materials may be useful in a broad range of biomedical applications, from depots for drug delivery, to scaffolds for cell delivery and tissue engineering.

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Figure 1. Synthesis of poly(HPMA)-g-CGGBeta11 graft copolymer and its self-assembly into a hydrogel via association of pendant β -sheet peptide strands.

Figure 2.

A. Temperature-dependent CD spectra of Beta11 peptide (the arrow indicates the change of spectrum upon temperature increase); B. Temperature-dependent CD spectra of poly(HPMA)-g-CGGBeta11 graft copolymer; C. Temperature effect on the secondary structure of Beta11 and poly(HPMA)-g-CGGBeta11, recorded as change in ellipticity at 218 nm; D. pH-Dependent CD spectra of Beta11 peptide; E. pH-Dependent CD spectra of poly(HPMA)-g-CGGBeta11 graft copolymer.

Figure 3. FTIR spectra (solid) and second-derivatives (dotted) of A. Beta11 peptide; B. poly(HPMA)-g-CGGBeta11 graft copolymer.

Figure 4.

A. Fluorescence emission spectra ($\lambda_{exc} = 440$ nm) of Beta11 peptide and poly(HPMA)-g-CGGBeta11 graft copolymer after addition of 50 μ M ThT. ThT alone was used as blank; B. CR binding assay; C. Differential spectra of (Beta11/CR)-CR and (poly(HPMA)-g-CGGBeta11/CR)-CR showing the points of maximum absorption.

Figure 5.

A. TEM image of negatively stained Beta11 fibrils; B. TEM image of negatively stained poly(HPMA)-g-CGGBeta11 fibrils; C. SEM image of freeze-dried 0.4 wt % Beta11 gel; D. SEM image of freeze-dried 0.4 wt % poly(HPMA)-g-CGGBeta11 gel (the inset is the image of the cross-sectioned copolymer gel).

Figure 6. Mean square displacement (MSD) as a function of lag time for 0.52 μm amidine-modified PS particles in water solutions/gels at different concentrations of A. Beta11 peptide; B. poly (HPMA)-g-CGGBeta11 graft copolymer.

Figure 7.

Frequency-dependent linear viscoelastic moduli for 0.52 μm amidine-modified PS particles in water solutions/gels of A. Beta11 peptide (solid symbols for loss modulus, G'' , and half-open red symbols for storage modulus, G'); B. poly(HPMA)-g-CGGBeta11 graft copolymer (open symbols for loss modulus, G'' , and half-solid red symbols for storage modulus, G').

Table 1
Peptide structures

	1	2	3	4	5	6	7	8	9	10	11
Pl 1-3 ¹³	CH ₃ CO-	Gln	Gln	Arg	Phe	Gln	Trp	Gln	Phe	Gln	Gln-Gln-NH ₂
Beta 11	CH ₃ CO-	Thr	Thr	Arg	Phe	Thr	Trp	Thr	Phe	Thr	Thr - Thr - Thr -NH ₂
CGGBeta11	H-Cys	Gly	Gly	Thr	Thr	Arg	Phe	Thr	Trp	Thr	Phe- Thr - Thr - Thr -NH ₂