Title: Design of a shear-thinning recoverable peptide hydrogel from native sequences and application for influenza H1N1 vaccine adjuvant

A self-assembling peptide was designed and synthesized by rationally combining two native sequences from an elastic segment of spider silk and a trans-membrane segment of human muscle L-type calcium channel. The peptide forms two distinct hydrogels in Ca²⁺ solution and acidic pH conditions. The shear-thinning, rapid-strength-recovering Ca²⁺ hydrogel has great potential for drug delivery and tissue engineering applications, for example, as an H1N1 influenza vaccine adjuvant.

As featured in:

Design of a shear-thinning recoverable peptide hydrogel from native sequences and application for influenza H1N1 vaccine adjuvant

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Received 29th January 2011, Accepted 25th June 2011
DOI: 10.1039/c1sm05157a

Peptide hydrogels are considered injectable materials for drug delivery and tissue engineering applications. Most published hydrogel-forming sequences contain either alternating-charged and non-charged residues or amphiphilic blocks. Here, we report a self-assembling peptide, h9e (FLIVIGSIIGPGGDGPGGD), designed by rationally combining two native sequences from an elastic segment of spider silk and a trans-membrane segment of human muscle L-type calcium channel. The turning segment GSHI of h9e promoted hydrogel formation in both Ca2+ solution and acidic pH conditions at water content greater than 99.5%. Although h9e Ca2+ hydrogel and h9e acidic hydrogel have the same sequence, they have distinct physical properties. The shear-thinning, rapid-strength-recovering h9e Ca2+ hydrogel was used as an H1N1 influenza vaccine adjuvant. The h9e adjuvant was biologically safe and improved immune response by ~70% compared with an oil-based commercial adjuvant.

Introduction

Using peptide hydrogels as injectable materials for drug delivery systems and tissue engineering applications has been an important discovery made over the past few decades. 1–6 Because of its high water content and polymer network, peptide hydrogel is a promising material for storage and transfer of proteins without significant loss of their biological activity. 7 A sol–gel transition occurs when peptide molecules self-assemble into a well-defined nanofibre network that traps water molecules. Because this transition occurs at specific temperature and pH, peptide hydrogel precursors may be injected into the body in the liquid phase and converted into hydrogel when physiological condition such as pH or temperature changed in vivo. 8–14 which is important for injectable applications. A special type of injectable hydrogel has shear thinning and rapid recovery characteristics. 12–14 The storage modulus of this kind of hydrogel decreases sharply under the shear force but recovers after the force is removed. Although the mechanism of this shear thinning and recovery phenomena is still under debate, this hydrogel-forming peptides have high potential for use as injectable materials for medical applications. 15

Within the last decade, many novel designed hydrogels have been achieved by amphiphilic peptides. 16–20 With a propensity of fiber structure, peptide monomers fabricate the nanofibre structure under the hydrophobic aggregation and the further crossing as a nanofibre network for hydrogel formation. On the other hand, the identified functional domains of native proteins were also selected and modified as potential models for peptide design in recent years. 21–23 Since these sequences exist in the biological system and were identified because of their potential for certain functions, rational design of peptides involving native functional domain sequences is a promising approach for development of functional biomaterials.

In this study, instead of choosing artificial designed model peptide monomer, we report a Ca2+ induced shear-thinning and rapid recoverable hydrogel forming peptide FLIVIGSIIGPGGDGPGGD (h9e) from rational combining two native sequences. The hydrophilic segment GPGGDGPGGD (eD2), designed from a β-spiral motif of spider flagelliform silk protein, has the potential to drive peptide monomer aggregation to nanofibre structure triggered by Ca2+. 24 The hydrophobic motif, FLIVIGSII (b9), was derived from the third trans-membrane segment of subunit IV in the dihydropyridine sensitive human muscle L-type calcium channel. 25 According to our previous study, solutions of peptides Kh5K (KKK-FLIVI-KKK) and Kh9K (KKK-FLIVIGSII-KKK) which have core FLIVI (h5) and FLIVIGSII (h9) flanked by tri-lysine segments are characterized by high adhesion shear strength and unusual biophysical properties. 26 The initial design of peptides examined in this study was performed by combining...
both h5 and h9 segments with eD2 as two amphiphilic peptides FLIVIGPGGDGPGGD (h5e) and h9e. The hydrophobic cores h5 and h9 were expected to accelerate the strength of nanofiber formation, a serial of peptides were synthesized by modifying the primary structure of h9e. The characters of h9e hydrogel were identified by morphological and structure observation and mechanical property tests in different environmental conditions.

Furthermore, the potential application of injectable h9e Ca2+ hydrogel was also tested. Adjuvant, for example, has been used for decades to improve the immune response to vaccine antigens. Current adjuvants include mineral salts, oil emulsions, particulate adjuvant, and microbial derivatives. Many of them have unacceptable side effects and lack biocompatibility. Some are biologically toxic. A large array of new vaccine candidates has been developed over the past years or will be introduced in the future to work against infectious, allergic, and autoimmune diseases and also for cancer and fertility treatment, which all require diverse new adjuvants with desirable functions and performance to successfully achieve new vaccine development and implementation. In this study, the h9e Ca2+ hydrogel was evaluated as adjuvant for H1N1 swine influenza virus killed vaccine.

Experiment

Peptide synthesis

Peptides were synthesized on a CEM Liberty microwave peptide synthesizer (CEM Corporation, Matthews, NC) according to the automated base-labile 9-fluorenlymethoxycarbonyl (Fmoc) strategy with Fmoc-protected amino acids (EMD Biosciences, San Diego, CA). Peptides were cleaved using 95% trifluoroacetic acid (Sigma-Aldrich, Milwaukee, WI), 2.5% triisopropylsilane (Sigma), and 2.5% deionized water. After synthesis, peptides were washed three times with anhydrous ether (Fisher Biotech, Fair Lawn, NJ), dissolved in acetonitrile and distilled (DI) water (50/50 v/v), and then freeze-dried. Molecular weight and purity of the synthesized peptide were confirmed by matrix-assisted laser desorption/ionization time-of-flight mass spectroscopy and high performance liquid chromatography (HPLC, Beckman Coulter, Inc., Fullerton, CA).

Hydrogel preparation

The synthesized peptide was dissolved in DI water to a concentration of 5 mM by adjusting the pH to 8.0–10.0 with 1 M NaOH (Sigma). The acidic h9e hydrogel was prepared by adjusting the pH to 4.0 with 1 M HCl (Sigma). The h9e Ca2+ hydrogel was prepared by adding CaCl2 to the basic h9e peptide solution (molar ratio of peptide and Ca2+ was 1 : 10, final pH was 7.0 to 9.0).

Transmission electron microscopy (TEM)

Peptide solutions were prepared on Formvar/carbon-coated 200-mesh copper grids (Electron Microscopy Sciences, Fort Washington, PA) and stained with 2% (w/v) uranyl acetate (Ladd Research Industries, Inc., Burlington, VT) for 60 s at ambient conditions before being imaged. The samples were imaged with a CM100 TEM (FEI Company, Hillsboro, OR) at 100 kV.

Laser scanning confocal microscope (LSCM)

Confocal fluorescence images of the h9e solution and hydrogels were taken on an LSM 5 PASCAL confocal microscope (Carl Zeiss Inc, Thornwood, NY) at an excitation wavelength of 543 nm. The fluorescent dye DiIC18 (dissolved in 100% EtOH 1 mg mL−1) was incorporated into the peptide molecules, and fluorescence was imaged with a 560LP filter.

Scanning electron microscope (SEM) analysis

Morphology of h9e Ca2+ hydrogel with 2.5 mM concentration was observed with backscatter electrons (BSE). The sample was mounted on a holder using a conductive adhesive and placed on a cold stage. The sample was cooled to −150 °C then warmed to and held at −30 °C. The chamber pressure was 40 Pa. Images were taken on a Hitachi S-3500N SEM (Hitachi Science Systems, Ltd., Hitachinaka, Japan) at an accelerating voltage of 15 keV with a Robinson backscatter detector.

Mass spectrometry (MS)

MS experiments were performed using LTQ-Orbitrap (Thermo Electron Bremen, Germany) equipped with an electrospray ionization source. Samples were injected through a pulled fused silica capillary (50 mm ID) at a flow rate of 0.3 to 0.5 mL min−1 using a spray voltage of 4 kV. The system was operated in the positive ion mode with a resolving power of 60,000 at m/z 400. MS/MS experiments were performed using a 2 to 3 amu isolation window. The collision energy was adjusted for each species to obtain ca. 70 to 90% fragmentation of the precursor ion. High-resolution mass analysis enabled unambiguous identification of the resulting fragments.

Stock solutions of peptides were prepared by dissolving 0.85 mg and 0.94 mg of h5e (MW 1370.6951) and h9e (MW 1740.9167), respectively, in 500 μL HPLC grade water and adding 60 to 80 μL of 0.25 M NaOH to obtain solutions with pH 8. Solutions for MS experiments were prepared by mixing 10 μL of the stock solution with 10 μL of 0.1 M CaCl2 and adding 200 μL of 50 : 50 (v:v) H2O/acetonitrile.

Circular dichroism (CD) experiments

The CD spectra of h9e acidic hydrogel, and Ca2+ hydrogel were recorded at ambient conditions using a Jasco J-815 spectrometer (Jasco Corporation, Tokyo, Japan). The concentration of samples was 1 mM (0.17 wt%). CD spectra were recorded from 190 to 260 nm with 1 nm bandwidth and 20 nm min−1 scanning speed, then averaged over two accumulations. Baselines were recorded using basic, acidic, and Ca2+ solutions without peptide.

Fourier transform infrared spectroscopy (FTIR)

The Ca2+ and acidic hydrogels of h9e were prepared and freeze-dried. The FTIR spectra were recorded on a PerkinElmer.
spectrum 400 FT-IR/FT-NIR spectrometer (PerkinElmer Inc, Waltham, MA) in the range of wavenumbers from 400–4000 cm⁻¹. The accumulation was 16 scans and the peaks were identified by deconvolution.

Rheology

The storage, G', and loss, G'', moduli of h9e acidic and Ca²⁺ hydrogels were determined on a rheometer system C-VOR 150 (Malvern instruments, Malvern, Worcestershire WR141XZ, United Kingdom) with a 20 mm diameter parallel plate geometry through frequency sweep (strain 1%, frequency 0.01 to 10 Hz, temperature 25 °C), amplitude sweep (strain 1 to 500%, frequency 1Hz, temperature 25 °C), and temperature profile (strain 1%, frequency 1Hz, Temperature 5, 20, 37, 50, 75, and 90 °C) measurements. The multiple amplitude sweep experiments were conducted to test the moduli recovery of peptide hydrogels. The time gap between every two tests was 10, 30, and 60 s for h9e Ca²⁺ hydrogel.

Animals and vaccination study

C57/BL6 mice (female, 8 wk old, 4 mice per group) were immunized twice in a 3-wk interval with killed H1N1 swine influenza virus antigen (Pfizer) with h9e or commercial adjuvant (Pfizer). Sera were collected from each mouse 2 weeks after the second immunization.

Antibody response analysis

Anti-swine H1N1 influenza virus-specific IgG1 were determined by enzyme-linked immunosorbent assay following the methods described by Skountzou et al. and Koutsonanos et al. with some modifications. Optical density (OD) was read at 450 nm. The results were expressed as an S/P ratio calculated as the mean OD of duplicate wells of each unknown serum divided by the mean OD of a positive control.

The HAI titer followed the WHO protocol. After proper treatments, heat-inactivated sera were serially diluted and pre-incubated at room temperature with 4 HA units/50 ml of H1N1 virus for 30 min. An equal volume of 0.5% chicken red blood cells was then added to each well and incubated at room temperature for 30 min. The HAI titer was read as the reciprocal of the highest dilution of serum that conferred inhibition of hemagglutination. The values were expressed as the geometric mean of each treatment group.

Results and discussion

The h5e and h9e were dissolved in 100 mM NaOH solution. Under TEM examination, short fiber integrations were observed in the h5e solution (Fig. 1a), while the h9e solution contained predominately dimers of needle-shape nanofibers of about 10 nm width (Fig. 1 e). Changing the pH value to acidic or adding Ca²⁺ solution made the h5e solution become cloudy with sedimentation. The h5e molecules aggregated into large insoluble particles that could be observed with the naked eye. However, the same process produced two different kinds of hydrogels in h9e solution. These significantly different behaviors between these two peptides were unexpected. Compared their primary structures, beside the hydrophobic core h5 and the fiber structure induced region eD₂, turning segment GSII, the only difference in the sequence of h5e and h9e, indicated a key role in hydrogel formation.

Mass spectrometry (MS) experiments were conducted to identify possible precursors of the peptide assembly and nanofiber crossing in a Ca²⁺ solution of h5e and h9e peptides (Fig. 1f). Mass spectrum obtained for both peptides was dominated by Ca²⁺ adducts indicating high affinity of h5e and h9e to calcium. MS/MS experiments were conducted to gain insight on the mode of binding of calcium to h5e and h9e peptides. In agreement with earlier work, fragmentation of peptide molecules cationized on calcium produces a number of backbone fragments including y-, b-, a-, z- and c-ions. It has been demonstrated that the a-ion formation is promoted by calcium binding and occurs C-terminal to the Ca²⁺ binding site. Examination of MS/MS spectra obtained for different calcium adducts of the h5e and h9e peptides (supplementary tables 1, 2) shows that in [M + Ca]²⁺ ion calcium is most likely coordinated by the carboxyl group of the internal D residue and solvated by the C-terminal D. Fragmentation behavior changed in an interesting way for [h9e + 2Ca]⁴⁺ (supplementary table 1). Cleavages indicative of Ca²⁺ binding were observed in the SI1 and GDGP regions, suggesting that although the first Ca²⁺ is bound to the internal D residue, the second one is coordinated by serine. Differences in Ca²⁺ binding capacity of the two peptides indicated the different assembly pathways. The tight Ca²⁺ binding h9e has more compact structure and less hydrophobic area exposure than h5e (Fig. 1g, h), which may explain the morphological difference of their supramolecular aggregation and why hydrogel formed in h9e but not h5e.

To further understand the contribution of each sequence region for hydrogel formation, we divided the h9e sequence into three regions: the relatively hydrophobic part FL1VI, the relatively hydrophilic part GPGDGPGD, and the critical GSII segment, and designed a serial of peptides by modifying each region (Table 1). The peptide h5SIIe designed by removing the glycine residue from the GSII segment exhibited the short fiber integrations in aqueous solution (Fig. 1b). Unlike hydrogel of h9e or insoluble particles of h5e, h5SIIe aggregated into gel-like particles in the presence of Ca²⁺ or in acidic solution. This phenomenon suggested that the conformation freedom provided by the glycine residue should be another critical factor for peptide correct assembly. For the further study, two relative inflexible segments, IIVI and PPD, were selected to replace GSII segment for peptide h5IIVIe and h5PPDe. IIVI is a hydrophobic and linear structure, but PPD is a sharp turning motif. The peptide h5IIVIe has similar morphology as h5e (Fig. 1c), and become insoluble particles in the presence of Ca²⁺ or in acidic solution. Surprisingly, the peptide h5PPDe was soluble in neutral water without any pH adjustment. The small amorphous morphology and spherical aggregation presented in TEM images of h5PPDe (Fig. 1d), indicate the spherical assembly of peptide molecules when the sharp turning h5PPDe fold the hydrophobic region and the hydrophilic region as a hairpin structure (Fig. 1f). Except h9e, none of these peptides formed hydrogel. Both the correct packing of peptide monomers with metal ions and the conformation freedom between the hydrophobic and hydrophilic segments of peptides played important roles for hydrogel forming capability.
In addition, two other peptides, L5GSIIe, h5GSIIK10 were designed by retaining the GSII motif and substituting the h5 segment of h9e with LLLLL or replacing the eD2 segment of h9e with KKKKKKKKKK, respectively. These two sequences formed viscous solution or hydrogel with low modules. The storage module of 5mM L5GSIIe is 21.6 ± 0.3 Pa in Ca2+ solution and that of 5mM h5GSIIK10 is 55.0 ± 0.9 Pa in basic pH, both of which were much smaller than the storage module of the h9e hydrogel, 1560.0 ± 13.0 Pa (h9e Ca2+ hydrogel) and 2863.7 ± 27.8 Pa (h9e acidic hydrogel), formed at the same concentration (Fig. 3a). Here, native functional sequences h5 which could provide the high adhesion shear strength and eD2 which has ion binding and fiber forming propensity display a great approach for the mechanical properties of this hydrogel. When we replaced both h5 and eD2 segments of h9e, the peptide L5GSIIK10 did not form hydrogel in aqueous solution. These findings suggest that h9e, designed from rational combination of two native segments, is a unique hydrogel-forming sequence.

The h9e formed hydrogels in Ca2+ solution as well as at acidic pH (Fig. 2a, d). The needle shape nanofibers (about 10 nm width)

<table>
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<th>Table 1</th>
<th>Sequences of synthesis</th>
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<tr>
<td>Peptide</td>
<td>FLIVI-GPGGDGGPPGD</td>
</tr>
<tr>
<td>h5e</td>
<td>FLIVI-GSII-GPGGDGGPPGD</td>
</tr>
<tr>
<td>h9e</td>
<td>FLIVI-IIVI-GPGGDGGPPGD</td>
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<tr>
<td>h5SIIe</td>
<td>FLIVI-GSII-GPGGDGGPPGD</td>
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<tr>
<td>h5IIVIe</td>
<td>FLIVI-IIVI-GPGGDGGPPGD</td>
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<td>h5PPD</td>
<td>FLIVI-IIVI-GPGGDGGPPGD</td>
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<td>h5SIIe</td>
<td>FLIVI-IIVI-GPGGDGGPPGD</td>
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<td>h5IIVIe</td>
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In aqueous solution, the h5e (a) h5SIIe (b) and h5IIVIe (c) integrations respectively, d. TEM image of micelle-like structure of h5PPD, e. needle shape nanofibres of h9e, f. MS fragments of h5e and h9e, g-i. assembly of amphiphilic peptides (g: linear peptide, h: peptide with flexible turning segment, i: peptide with sharp turning; blue: hydrophobic segment, red: hydrophilic segment, yellow: turning segment).
were extended and crossed as fiber-networks in Ca²⁺ solution to form a soft-gel (Fig. 2b). In contrast, h9e formed the hard hydrogel in acidic conditions, the nanofibers of which aggregated parallel as thicker fibers and entangle for a network (Fig. 2e).

Under the observation of LSCM, the microporous morphology presented in h9e Ca²⁺ hydrogel (Fig. 2c): the nanofibres crossed each other and aggregated like nanoparticles at the crossing points, while, a tangle of more robust fibers was observed in h9e acidic hydrogel (Fig. 2f). The pores of h9e hydrogel were bigger and more obvious in Ca²⁺ than in acidic conditions as shown in SEM images (ESI† Fig. 1a, b). The circular dichroism (CD) spectra (Fig. 2g) suggested that h9e adopted predominantly β-sheet in Ca²⁺ solution, whereas a more random structure was detected in the acidic solution, which has much lower intensity as well, suggesting the disordered chain conformations of h9e acidic hydrogel. Fig. 2h shows amide I and amide II region of FTIR spectra of h9e Ca²⁺ and acidic hydrogels (solid: h9e acidic hydrogel, open: h9e Ca²⁺ hydrogel).
observed in the acidic h9e hydrogel. Normally, stable chain conformations are considered as essential factors in the formation and strength of hydrogels, however, h9e acidic hydrogel, which even have greater strength than h9e Ca2+ hydrogels at concentration lower than 5 mM (Fig. 3a), does not follow this common rule.

A storage moduli (G’) of the h9e acidic gel is about 10 times higher than that of the h9e Ca2+ gel at 2.5 mM peptide concentration (Fig. 3a). The difference in G’ between these two hydrogels became smaller as peptide concentration increased. For example, at 10 mM, G’ of the h9e Ca2+ gel was about 9000 Pa, which was even higher than that of the h9e acidic gel (about 8000 Pa). The dramatically increase of G’ of h9e Ca2+ gel with peptide concentration may be contributed by its highly order secondary structure formation which made the self-assembly of peptides more compact and nanofibers crossing more tightly at higher concentration. The effect of the molar ratio of Ca2+ and peptide on the G’ of h9e Ca2+ gel was also tested (ESI† Fig. 2) and the G’ was found to be more stable when the ratio higher than 1. In a temperature profile test, G’ of the h9e Ca2+ hydrogel increased 10 fold as temperature increased from 5 to 90 °C, however, G’ of the h9e acidic hydrogel decreased as temperature increased and dropped to 1,000 Pa at 75 °C (Fig. 3b). The G’ of h9e Ca2+ hydrogel was reversible based on the changing of temperature within the range from 2 to 80 °C (Fig. 3c). The reversibility of the storage moduli with temperature fluctuation was not observed in the h9e acidic hydrogel. The shear thinning and rapid recovery of mechanical strength was found only in the h9e Ca2+ hydrogel (Fig. 3d). Hydrogels underwent a serial of amplitude sweep tests. There was a short delay after every two test cycles. Gel became like pure liquid (G” > G’ ≈ 0) under 500% strain oscillation. After 10 s of the first cycle, 75 to 80% of the hydrogel strength was recovered. Percentage of strength recovery increased as the delay time increased and reached 100% recovery by 60 s. Under the same process, the strength of the h9e acidic hydrogel did not recover in this short time period (Fig. 3e). The multiple recovery properties and short recovery time suggest the h9e Ca2+ hydrogel has large potential for biomedical application.

This paper is our first report of two types of hydrogels with distinct mechanical properties created from a peptide with the same primary structure. According to published studies, changing an amino acid in certain positions could result in peptide hydrogels with different mechanical strength, thermal response, and recovery property because these changes in primary structures facilitated molecular folding and cross-linking of peptide fibers. The distinct properties of these two h9e hydrogels suggest that h9e molecules could undergo different molecular assembly and nanofiber cross-linking controlled by different external parameters despite having the same primary structure.

Ions Na+, Mg2+, and Zn2+ were also studied with h9e. In these three ion solutions, h9e formed hydrogels with different G’ (ESI† Fig. 3a). The h9e Na+ hydrogel had physical properties similar to those of the h9e Ca2+ hydrogel (ESI† Fig. 3b, c). However, h9e formed a soft hydrogel in Mg2+ solution. In Zn2+ solution, h9e formed a hard hydrogel as it did in h9e acidic gel. The rapid shear strength recovery property was not found in h9e Mg2+ or h9e Zn2+ hydrogels (ESI† Fig. 3d, e).

The h9e Ca2+ hydrogel was applied as an adjuvant for vaccine antigen delivery and showed significantly higher efficiency than a commercial adjuvant. Mice immunized with h9e-adjuvanted vaccine or commercial vaccine did not show any abnormality and remained healthy before they were euthanized for sera
collection. No injection site reaction (redness and swelling) was observed in vaccinated mice. Adjuvanticity of h9e Ca^{2+} hydrogel was determined by immunizing mice with killed H1N1 swine influenza virus in the presence or absence of h9e Ca^{2+} hydrogel and a commercial adjuvant, which was used as a positive control. As shown in Fig. 4a, the mean hemagglutination inhibition (HAI) titer in the sera from mice immunized with h9e Ca^{2+} hydrogel and killed H1N1 virus antigen was 1020, which was about 70% higher than the HAI titer of 600 observed in mice immunized with commercial vaccine. HAI activity was not detected in sera from mice treated with antigen in the absence of an adjuvant.

We also measured the effect of h9e Ca^{2+} hydrogel on production of antigen-specific IgG1 antibody response. As shown in Fig. 4b, a positive antigen-specific antibody response (S/P ratio >0.4) was observed in mice immunized with antigen plus h9e Ca^{2+} hydrogel or commercial adjuvant. Mice immunized with killed antigen alone did not produce any detectable H1N1-specific IgG1 antibody (data not shown). There was no significant difference between h9e Ca^{2+} hydrogel and commercial adjuvant in ability to induce an H1N1-specific IgG1 antibody response.

Our studies provide the first evidence that h9e Ca^{2+} hydrogel can be a safe, efficacious adjuvant for H1N1 swine influenza virus killed vaccine. Known adjuvants usually render adjuvanticity by directly stimulating antigen-presenting cells and other immune cells or by controlling the release of antigens from the injection site. It is possible the hydrogel network that controlled the antigen release, or the major segments of h9e, which were selected from native functional protein, activate immune cells directly through a specific cell surface receptor. It is equally possible that h9e and killed H1N1 viruses form nanoparticles as was shown in the LSCM image (Fig. 2c). It is well-established that microparticles can function as adjuvants to promote immune responses. For example, anionic microparticles coated with recombinant p55 gag protein from HIV-1 elicited strong cell-mediated immunity as well as antibody response in mice. Future studies will determine whether h9e can directly regulate immune cell functions and whether its adjuvanticity is mediated solely through controlling the release of antigens from the molecular platform of h9e-antigen interaction.

Conclusion

We rationally used two native functional sequences from spider silk and a trans-membrane motif of human muscle calcium channel to design and synthesize novel peptides that were sensitive to Ca^{2+}. However, eD2 alone or tailored with hydrophobic segments (i.e., h5 = FLIVI) was not able to form hydrogels. The turning function of GSII played a key role, altering the molecular assembly pathways of h9e for hydrogel formation. In acidic conditions, h9e formed hard hydrogels that had a storage modulus (G') 10 times stronger than that of the hydrogel formed in Ca^{2+} solution (at 2.5 mM peptide concentration). The G' of h9e acidic hydrogel was weakened upon heating, whereas the G' of h9e Ca^{2+} hydrogel increased as temperature increased and was reversible in the temperature range of at 2 to 80 °C. The h9e Ca^{2+} hydrogel was shear-thinning and had 100% recovery within 1 min. These distinct physical properties between h9e acidic hydrogel and h9e Ca^{2+} hydrogel suggest that a peptide’s molecular assembly pathways and degree of nanofiber cross-linking could be induced by external parameters such as pH and metal ions. The h9e has great potential for drug delivery and tissue engineering applications. The adjuvant prepared using the h9e acidic hydrogel and h9e Ca^{2+} hydrogel suggest that a peptide’s molecular assembly pathways and degree of nanofiber cross-linking could be induced by external parameters such as pH and metal ions. The h9e has great potential for drug delivery and tissue engineering applications.

Acknowledgements

We thank Dr Dan Boyle (Department of Biology, Kansas State University) for TEM and LSCM images, Dr Michal Zolkiewski (Department of Biochemistry, Kansas State University) for CD use, Dr Duy Hua (Department of Chemistry, Kansas State University) for HPLC use, and the University of Kansas Mass Spectrometry Lab for mass spectrometric analysis. Funding for this work was provided by the Targeted Excellence Program and Center for Biobased Polymers by Design at Kansas State University. Contribution no. 10-127-J from the Kansas Agricultural Experiment Station. MS/MS experiments were performed at the W. R. Wiley Environmental Molecular Sciences Laboratory (EMSL), a national scientific user facility sponsored by the U.S. DOE of Biological and Environmental Research and located at the Pacific Northwest National Laboratory.

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