Published in final edited form as:

Biomaterials. 2010 November ; 31(32): 8475–8483. doi:10.1016/j.biomaterials.2010.07.068.

Immune responses to coiled coil supramolecular biomaterials

Jai S. Rudra^a, Pulak Tripathi^c, David A. Hildeman^c, Jangwook P. Jung^a, and Joel H. Collier^{a,b,*}

^a Department of Surgery, University of Chicago, 5841 S. Maryland Ave., Chicago, IL 60637

^b Committee on Molecular Medicine, University of Chicago, 5841 S. Maryland Ave., Chicago, IL 60637

^c Division of Immunobiology, Cincinnati Children's Hospital, and the Department of Pediatrics, University of Cincinnati, OH 45229

Abstract

Self-assembly has been increasingly utilized in recent years to create peptide-based biomaterials for 3D cell culture, tissue engineering, and regenerative medicine, but the molecular determinants of these materials' immunogenicity have remained largely unexplored. In this study, a set of molecules that self-assembled through coiled coil oligomerization was designed and synthesized, and immune responses against them were investigated in mice. Experimental groups spanned a range of oligomerization behaviors and included a peptide from the coiled coil region of mouse fibrin that did not form supramolecular structures, an engineered version of this peptide that formed coiled coil bundles, and a peptide-PEG-peptide triblock bioconjugate that formed coiled coil multimers and supramolecular aggregates. In mice, the native peptide and engineered peptide did not produce any detectable antibody response, and none of the materials elicited detectable peptide-specific T cell responses, as evidenced by the absence of IL-2 and interferon-gamma in cultures of peptide-challenged splenocytes or draining lymph node cells. However, specific antibody responses were elevated in mice injected with the multimerizing peptide-PEG-peptide. Minimal changes in secondary structure were observed between the engineered peptide and the triblock peptide-PEG-peptide, making it possible that the triblock's multimerization was responsible for this antibody response.

Keywords

self-assembly; immunogenicity; tissue engineering; regenerative medicine

INTRODUCTION

Biomaterials constructed from self-assembling peptides and peptide-polymer conjugates are receiving increasing attention for a variety of biomedical and biotechnological applications, including tissue engineering and regenerative medicine [1,2]. Recently developed strategies have included those based on β -sheet fibrillizing peptides [3-6], peptide-amphiphiles [7,8],

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*Corresponding Author Joel H. Collier, Ph.D. Assistant Professor Department of Surgery and Committee on Molecular Medicine University of Chicago 5841 S. Maryland Ave. MC 5032 Chicago, IL 60637 collier@uchicago.edu.

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short peptides with aromatic groups [9], and α -helical coiled coil peptides [10-13]. Collectively, these materials have shown considerable promise for applications in regenerative medicine [2,8,14,15], drug delivery [16-19], and defined cell culture matrices [6,11]. At the same time, the structural determinants of their immunogenicity are not well understood, and strategies for modulating immune responses against them have not been significantly developed despite the importance of such considerations in their ultimate clinical use.

Peptides are generally poor immunogens and usually require coadministration with strong adjuvants to elicit antibody responses; however, their immunogenicity can increase significantly upon supramolecular assembly [20,21]. Aggregation can render native human proteins immunogenic [22,23], and spatially repeated epitopes can raise strong antibody responses without T cell help [24,25]. While no studies to our knowledge have previously investigated the immunogenicity of self-assembling peptide biomaterials based on coiled coils, recent work with self-assembling β -sheet peptides illustrate how divergent immune responses against peptide biomaterials can be. Previously, several β -sheet fibrillized biomaterials have shown minimal or immeasurable immunogenicity [7,8,14,26-28], including Q11, a β -sheet fibrillizing peptide under investigation in our laboratory [21,26]. However, Q11 peptides functionalized with a peptide sequence from ovalbumin containing known T cell and B cell epitopes can elicit very strong antibody responses even in the absence of any adjuvant [21]. For Q11, the presence or lack of a relevant epitope on the surface of the fibrils is a determining factor that switches the material from being non-immunogenic to being highly so, providing guidance both for minimizing immunogenicity in tissue engineering applications or for purposefully inducing strong antibody responses in immunotherapies.

In the present work, we evaluated structural features that modulate immunogenicity for α -helical coiled coil-based biomaterials. Peptides, proteins, and peptide-polymers that form networks via coiled coil multimerization provide several advantages over polymer matrices or biologically sourced ECM proteins, including compositional definition, control over network topology [29-34], and stimulus-responsiveness [35-39]. In contrast to β -sheet fibrillizing peptides, coiled coils are found in many native extracellular matrix proteins [40], and well-established design rules can be exploited to engineer stable oligomerized bundles [41]. To form scaffolds or hydrogels, coiled coils have been employed as physical cross-linkers for polymer networks [36,42], as oligomerizing blocks in designed proteins [12,33,38], and as components of long helical nanofibers [10,43]. We have previously designed hydrogels based on coiled coil peptides from human fibrin, the principal component of the hemostatic plug [13]. The original motivation of this work was to produce synthetic peptide gels with significant homology to native human proteins in order to minimize immunogenicity. However, to stabilize the coiled coils and link them into extended networks, several steps were necessarily taken that caused the materials to diverge from the native protein structure. Amino acid substitutions were made at residues forming the hydrophobic core of the coiled coil, and these peptides were conjugated to polyethylene glycol (PEG) in a triblock peptide-PEG-peptide configuration [13]. Self-assembly of this triblock peptide-polymer affords well-folded viscoelastic hydrogels, but the extent to which the amino acid substitutions and supramolecular assembly may influence the material's immunogenicity is not known. Moreover, while it is generally known that aggregation can raise the immunogenicity of therapeutic proteins [22,23], PEG conjugation can be employed to minimize immune responses [44,45], making it unclear how these seemingly opposing influences might play out in any immune responses to self-assembling PEG-peptides. In the present study, we sought to resolve these questions by investigating the immunogenicity of the triblock and its intermediates in mice. Owing to the use of a mouse model, we produced a new series of peptides and peptide-polymers following the same design steps as the

previously reported triblock [13], but using peptides from mouse fibrin rather than human fibrin (Figure 1). Clarification of the molecular features that contribute to coiled coil-based biomaterials' immunogenicity will be important information for their ultimate clinical application given the frequent use of designed or non-native peptide sequences in them [13,30,34,37,38,43] and the current lack of information regarding their immunogenicity.

MATERIALS AND METHODS

Peptide and peptide-polymer synthesis

Peptides γ 51-85 (native peptide), γ KEI, and Cys- γ KEI were purchased from Sigma Genosys at 95-99% purity (sequences in Figure 1 and Table 1). The peptide-PEG-peptide triblock conjugate was synthesized using maleimide-thiol chemistry as previously reported [13]. Briefly, to a 3 mg·mL⁻¹ stock solution of the Cys- γ KEI peptide in phosphate buffered saline, 2 equivalents of PEG-dimaleimide (nominal 3.4 kDa PEG spacer, SunBio) were added in 0.25-equivalent increments over the course of 24 h at pH 6.5. The reaction was monitored using a Varian Prostar HPLC system using an acetonitrile/water gradient. When the reaction of Cys- γ KEI was complete, the mixture was purified on a C18 semipreparative column, on which the triblock eluted between 52-53% acetonitrile. The acetonitrile was removed by centrifugal evaporation. Samples were lyophilized and stored as powders at -20 °C.

Mass spectrometry

The triblock peptide-polymer was analyzed using ESI-MS on a Waters Micromass Q-TOF II mass spectrometer. The peptides were injected in 50% acetonitrile/water with 0.1% folic acid, and the acquired data were processed using MassLynx 4.0 software. The unconjugated peptides were analyzed using MALDI-MS, performed on an Applied Biosystems Voyager 6187 instrument using α -cyano-4-hydroxycinnamic acid as the matrix. Positive ions were analyzed in linear mode. Mass-to-charge ratios were as follows: γ 51-85 m/z calcd: 4131.5; found: 4131.0. γ KEI m/z calcd: 4270.8; found: 4271.0. Cys- γ KEI m/z calcd: 4374.0; found: 4373.0. Triblock average m/z calcd: 12,144; max peak found: 12,270.7.

Circular dichroism spectroscopy

An AVIV 215 circular dichroism spectropolarimeter was used with 0.1 cm path length quartz cells. Stock solutions were prepared by dissolving the peptides in ultrapurified water (Millipore Milli-Q system), and peptide concentrations were determined by Tyr absorbance at 274 nm. Stocks were diluted to a working concentration of 20-80 μ M in phosphate buffered saline (PBS, 0.2 g/L KCl, 0.24 g/L KH₂PO₄, 8 g/L NaCl, 1.44 g/L Na₂HPO₄), and pH was adjusted to 7.4. Under the solution conditions described, adequate signal strength was observed at wavelengths greater than 200 nm. Samples were scanned in triplicate at room temperature.

Analytical ultracentrifugation (AUC)

Sedimentation velocity experiments were performed as previously reported [13] on a Beckman XL-I analytical ultracentrifuge (Beckman Coulter, Palo Alto, CA). Prior to centrifugation, samples were dialyzed overnight against PBS, and the dialysate was used as the reference buffer. Samples were centrifuged at 48,000 rpm at 20 °C. The peptide γ KEI and the triblock were analyzed at a concentration of 1 mg/ml in PBS at 230 nm. The analysis software SEDFIT was used to fit the velocity data, determine continuous size distributions, and evaluate the oligomerization states of the peptides [46]. For γ KEI, the frictional ratio was 2.6, and for the triblock it was 1.7, indicating somewhat rod-shaped species in both cases. For all samples, $s_{\min}$ was 0.1, $s_{\max}$ was 20, and the confidence level was 95%.

Peptide cytotoxicity

A standard MTS (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium) assay was utilized to evaluate peptide cytotoxicity (Promega, Madison, WI, cat# G3582). Primary human umbilical vein endothelial cells (Lonza, Switzerland), were seeded at a density of $8,850 \text{ cells}\cdot\text{cm}^{-2}$ in 96-well plates, cultured to confluence over 24 h in endothelial cell growth medium-2 (EGM-2, Lonza, Switzerland), and then incubated for 24h in EGM-2 containing 0.01, 0.1 or $1 \text{ mg}\cdot\text{mL}^{-1}$ of the γ 51-85, γ KEI, or triblock peptides. After incubation, the medium was replaced, MTS reagent was applied for 4 h, and absorbance of the media at 490 nm was measured using a microplate reader. Controls included cultures without peptide and cultures that were fixed with absolute ethanol. All groups contained 5 replicates.

Animals and immunizations

C57BL/6 mice (6 wks, female) were purchased from Taconic Farms and allowed to acclimate to their new environment in the Cincinnati Children's Hospital animal facility prior to receiving peptide immunizations. All peptides used in the animal studies were found to contain $\leq 0.15 \text{ EU}\cdot\text{mL}^{-1}$ endotoxin by LAL chromogenic endpoint assay (Lonza), well within acceptable limits [47] (native peptide, $0.15 \text{ EU}\cdot\text{mL}^{-1}$; γ KEI, $0.12 \text{ EU}\cdot\text{mL}^{-1}$; triblock, $0.05 \text{ EU}\cdot\text{mL}^{-1}$). All immunizations were prepared under sterile conditions. For injections, peptides and peptide-polymers were dissolved in sterile PBS at a concentration of $1 \text{ mg}\cdot\text{mL}^{-1}$ immediately prior to immunizations. Mice were immunized subcutaneously in the back with $100 \mu\text{L}$ of peptide formulation ($100 \mu\text{g}$ of peptide) and boosted at the end of 4 weeks with $50 \mu\text{g}$ of material. Positive control mice received the peptide AWGALANWAVDS (2W1S), a known MHC class II peptide [48], in complete Freund's adjuvant (CFA). They were boosted with the same peptide in incomplete Freund's adjuvant (IFA). Negative control mice received injections of PBS only. One week after boosting, blood was collected through the retroorbital sinus, the animals were sacrificed, and their spleens and draining lymph nodes were harvested. The blood samples were allowed to clot, and the serum was removed by centrifugation at $3,000 \text{ g}$ for 10 min. Sera were aliquoted and stored at -80°C until analysis. In all animal work, institutional guidelines for the care and use of laboratory animals were strictly followed under a protocol approved by the Institutional Animal Care and Use Committee of Cincinnati Children's Hospital.

Antibody responses

Antibody levels in mouse sera were measured by ELISA. High-binding ELISA plates (Costar cat #9018) were coated with a $20 \mu\text{g}\cdot\text{mL}^{-1}$ solution of the immunizing peptide in PBS at 4°C overnight. Washing steps were performed with 0.5% (v/v) Tween-20 in PBS, and blocking was performed with 1% BSA (Sigma cat #A3803) in PBST (PBST-BSA) for 1 h at room temperature. Sera were diluted 1:100 in PBST-BSA and added to the sample and control wells. The primary antibody was peroxidase-conjugated goat anti-mouse IgG (H+L), applied at 1:5000 dilution for 1 h (Jackson Immuno Research, cat #115035003). Plates were developed for 20 min using TMB substrate (eBioscience CA, cat #00-4201-56). Fifty microliters of stop solution ($1 \text{ M H}_3\text{PO}_4$) was added, and absorbance was measured at 450 nm (Spectramax M5 plate reader).

Responses of Splenocytes and Lymph Node Cells

Spleens and the draining lymph nodes of the immunized mice were pressed through $70 \mu\text{m}$ cell strainers, and isolated splenocytes and lymphocytes were collected. Red blood cells were lysed using ACK buffer ($150 \text{ mM NH}_4\text{Cl}$, 10 mM KHCO_3) and washed twice with balanced salt solution (BSS, 5.5 mM Dextrose , $0.44 \text{ mM potassium phosphate}$, $1.33 \text{ mM sodium phosphate}$, $1.7 \text{ mM calcium chloride}$, $5.3 \text{ mM potassium chloride}$, $136.8 \text{ mM sodium chloride}$).

chloride, 0.8 mM magnesium sulfate, 1 mM magnesium chloride, and 2 mL of 0.5% phenol red). Splenocytes or lymphocytes (10^6 cells/well) were plated in complete T cell medium (S-MEM supplemented with 3.75 mM dextrose, 0.9% L-glutamine, 0.6% essential amino acids, 1.26% non-essential amino acids, 0.9% sodium pyruvate, 9 mM sodium bicarbonate, 95 μ M gentamycin, 140 μ M penicillin-G, 60 μ M streptomycin sulfate, 44 μ M 2-mercaptoethanol, and 10% fetal bovine serum) containing 10 μ g·mL⁻¹ immunizing peptide or no peptide. Cultures from negative control mice (PBS-injected) received no challenging peptide. After 24 h incubation at 37 °C, interferon- γ (IFN- γ), and IL-2 concentrations were measured in the culture medium using a sandwich ELISA and methods similar to those previously reported [21] (see Supporting Information for detailed methods). Capture antibodies were affinity-purified rat anti-mouse IFN- γ (IgG1, eBioscience #14-7312-85) and affinity-purified rat anti-mouse IL-2 (IgG2a, eBioscience #14-7022-81). Cell culture supernatants were applied at a 1:10 dilution to plates coated with the capture antibodies, and bound IFN- γ and IL-2 were detected with biotin-conjugated detection antibodies (biotinylated rat anti-mouse IFN- γ , IgG1, eBioscience cat #13-7312-81; and biotinylated rat anti-mouse IL-2, IgG2b, eBioscience cat #13-7021-81, respectively). Standard curves were constructed using recombinant mouse IFN- γ and IL-2 (eBioscience cat #39-8311 and #39-8021, respectively).

Statistical analysis

Statistical Analysis was performed by ANOVA with Tukey's post hoc comparison, and p-values < 0.05 were considered significant.

RESULTS

Peptide Design

To investigate structural aspects that modulate the immunogenicity of self-assembling peptides based on coiled coils, we designed and synthesized a set of molecules useful for this purpose (Table 1, Figure 1). The design process, analogous to a system previously reported for human peptide sequences [13], consisted of: 1) selecting a sequence of amino acids in the coiled coil domain of mouse fibrin, 2) substituting amino acids making inter-helix contacts in the coiled coil to stabilize folding outside the context of the native protein, and 3) conjugating the modified peptide to polyethylene glycol (PEG) in a triblock peptide-PEG-peptide arrangement to provide materials capable of oligomerizing into extended supramolecular networks (Figure 1). Intermediates representing each of these steps were synthesized for immunological evaluation.

The peptide γ 51-85 (Figure 1a, hereafter referred to as the “native” peptide) was identified in the γ chain of mouse fibrinogen, spanning analogous residues to those previously investigated in the human form of this protein [13]. The native mouse peptide was expected to have poor coiled coil forming ability owing to its buried polar residues in putative **a** positions of the (abcdefg)_n heptad repeat (see helical wheel projection in Figure 1d), its imperfect charge-charge pairings between residues in **e** and **g** positions, and its similarity to the analogous native human peptide, which also adopted a random structure in solution [13]. To produce a new peptide with improved coiled coil folding, a modified version of the native peptide was designed (γ KEI, Figure 1b, Table 1, helical wheel projections in Figure 1e, f). In γ KEI, all residues in putative **a** and **d** positions were substituted with isoleucine. Also, substitutions were made for one residue in an **e** position, two residues in **g** positions, and one residue in a **c** position. The **e** and **g** position substitutions were made to promote inter-helical electrostatic interactions, and the **c** position substitution was made to adjust the peptide's net charge. In total, 14 of 35 residues were substituted, and no amino acids in the putative **b** or **f** positions were changed. Owing to these modifications, it was expected that γ KEI would be able to form coiled coil bundles based on the hydrophobic packing of

residues in the **a** and **d** positions, the numerous electrostatic interactions between residues in **e** and **g** positions, and its similarity to the human fibrin analog (sequence in Table 1), which forms well-ordered coiled coil bundles [13].

To produce more extensively oligomerized supramolecular structures beyond discrete coiled coil bundles, a peptide-PEG-peptide triblock molecule was produced from γ KEI (Figure 1c). A cysteine residue was added to the N-terminus of the γ KEI peptide, which allowed conjugation to bifunctional PEG-(maleimide)₂ with an average molecular weight of 3,400 Da. The identity of the triblock peptide was confirmed by ESI mass spectroscopy, which showed an average molecular weight of 12,270 Da and a characteristic 44 Da spacing reflective of the polydispersity of the PEG central domain (Figure 1g).

Peptide folding and supramolecular assembly

The folding behavior of the peptides in PBS at neutral pH was determined by circular dichroism spectroscopy. The secondary structure adopted by the native, γ KEI, and triblock peptide at two different concentrations, 20 μ M and 40 μ M, is shown in Figure 2 (2a and 2b respectively). The native peptide displayed a disordered structure at every concentration investigated (Figure 2). In contrast, both γ KEI and the triblock exhibited strong helical character, with CD minima at 222–224 nm and 210 nm. Spectra for γ KEI and the triblock were nearly identical to each other, both at 20 μ M and at 40 μ M (Figure 2). This indicated that PEG conjugation did not significantly alter the folding of γ KEI. The magnitudes of the molar ellipticities for γ KEI and the triblock were somewhat smaller than expected for a peptide adopting a 100% helical structure, as was also the case for the analogous human peptide and triblock [13]. Previous sedimentation equilibrium experiments with the human peptide suggested that this was ascribable to regions of the coiled coil bundles that were unfolded rather than to the presence of a population of fully unfolded peptides, as no peptides existed in a monomeric state. It is likely that this remains true for the mouse γ KEI peptide owing to its similarity to the human γ KI (Table 1).

To investigate the supramolecular oligomerization of γ KEI and the triblock, analytical ultracentrifugation (AUC) was employed. These measurements were performed at the same peptide concentration as the subsequent mouse immunizations (1 mg·mL⁻¹), providing an indication of the oligomerization state of the material actually delivered in vivo. Figure 3 shows the sedimentation velocity $c(s)$ profiles for the γ KEI peptide (dashed line) and the triblock (solid line). The oligomerization levels of the peptides were calculated from the estimated molecular weights and are indicated by the arrows. It was found that the peptide γ KEI sedimented as a mixture of two species with estimated molecular weights close to dimers for the smaller oligomer and between tetramers and pentamers for the larger oligomer (Figure 3, dashed line). In previous work, the human γ KI sedimented as dimers and tetramers [13], which was additionally confirmed with sedimentation equilibrium experiments. For both γ KEI (mouse) and γ KI (human), it is not known whether the helices existed in a parallel or antiparallel arrangement, but owing to the strongly helical character of the CD spectra, the oligomerization observed by AUC, and the pattern of charged residues placed in **e** and **g** positions (Figure 1f), the most likely structures for these peptides would be parallel coiled coil bundles. Notably, no oligomers in addition to these two discrete bundles were observed for γ KEI (inset, Figure 3, dashed line).

Given that γ KEI formed discrete bundles, it was anticipated that the triblock would form even higher order oligomers, simply on the basis of topological considerations. However, given the possibility that the PEG chains could alter or prevent oligomerization, AUC was conducted to determine the specific oligomerization behavior. Like γ KEI, the triblock also formed discrete bundles at 1 mg·mL⁻¹ in PBS, but in addition it also formed higher-order supramolecular structures (Figure 3, solid line). A large main peak was observed in the

sedimentation velocity $c(S)$ distribution, with an estimated molecular weight near that of two associated triblock molecules (triblock dimer). There are several possible conformations that could be taken by such a dimer of triblock molecules, including a four-helix bundle, a three-helix bundle with one peptide dissociated, a two-helix bundle with one peptide from each molecule dissociated, or a pair of two-helix bundles connected by two PEG chains. Given the preference of γ KEI to associate rather than form monomers in solution, the conformations possessing single helices would seem unlikely. In comparison with γ KEI, the most prominent feature of the triblock $c(S)$ distribution was the presence of higher molecular weight species, including 12-mers, 18-mers, 30-mers, 50-mers, and a significant amount of larger assemblies (Figure 3, solid line). The formation of these oligomers by the triblock was in striking contrast with the behavior of the unconjugated γ KEI peptide, which did not form any of these larger species.

Immune Responses

Immune responses were assessed in C57BL/6 mice. Prior to immunizations, the endotoxin levels and in vitro cytotoxicity of all peptides were measured. All endotoxin levels were below < 0.15 EU/mL, well below acceptable levels [47], and none of the peptides were cytotoxic in cultures of HUVECs at any concentration tested, including $1 \text{ mg} \cdot \text{mL}^{-1}$, which was the concentration also used for immunizations (Figure 4). These results enabled the interpretation of immune responses without any confounding effects from endotoxin contamination or any underlying cytotoxicity.

Mice were immunized with $100 \mu\text{g}$ of the native peptide, γ KEI, or the triblock peptide, with a boost of $50 \mu\text{g}$ of material after 28 days. The concentration injected, $1 \text{ mg} \cdot \text{mL}^{-1}$, was kept well below the concentration required for gelation of the triblock [13] in order to minimize any differences in gross solution properties (e.g. viscosity) between groups. One week after boosting, sera, lymph node cells, and splenocytes were collected. Anti-peptide IgG was measured in the sera, and the production of IL-2 and IFN- γ was measured in cultures of peptide-challenged lymph node cells and splenocytes. The antibody responses to the mouse peptides, compared to the positive control peptide 2W1S administered in CFA, are shown in Figure 5. None of the experimental peptides were given in CFA. The strongest antibody responses were produced by the mice immunized with the positive control peptide in complete Freund's adjuvant (CFA). In this group, all mice developed similarly strong antibody responses. Both the native peptide and γ KEI showed no antibody production, having minimal ELISA absorbances that were statistically indistinguishable from those of mice injected with PBS only. Poor immunogenicity is commonly observed for short, soluble peptides, which generally need to be delivered with adjuvants to elicit significant antibody responses [49]. In contrast, the triblock delivered without adjuvant raised a moderate antibody response that was not as great as the positive control, but that was significantly greater than the negative control, the native peptide, or γ KEI (Figure 5). All mice in the group injected with the triblock molecule developed measurable antibody responses, with the most strongly responding mouse developing a response that was more than half as strong as that elicited by the positive control. To eliminate the possibility that the differences in ELISA measurements could have been attributed to variable binding of the peptides to the ELISA plate, cross-reactivity experiments were performed. It was found that ELISA plates coated with the triblock and incubated with sera from mice injected with the native peptide did not produce high backgrounds or otherwise anomalous readings (Figure S1). The ELISA readings for the other non-corresponding groups, including native coat/triblock serum and native coat/ γ KEI serum were similarly negligible, making it unlikely that any of the peptides bound to the plate with significantly different efficiencies to produce variable measurements.

The involvement of T cells in the observed immune responses was investigated by measuring the production of IFN- γ and IL-2 in cultures of splenocytes and lymph node cells from the immunized mice, as shown in Figures 6 and 7. Upon stimulation with the immunizing peptide, splenocytes or lymph node cultures containing populations of peptide-reactive T cells would be expected to produce one or both cytokines. In Figures 6 and 7, IFN- γ and IL-2 production are shown in cultures of splenocytes from immunized mice that were either challenged with peptide in culture (hatched bars) or left unchallenged (white bars). Compared to positive controls (+ ctrl, 2W1S in CFA), neither IFN- γ nor IL-2 was produced in the groups that were immunized and challenged with the native peptide, γ KEI, or the triblock (Figures 6 and 7). Instead, peptide-challenged cultures were similar both to non-challenged cultures as well as the negative control (– ctrl, mice injected with PBS and challenged in vitro with PBS only). Splenocyte cultures from the positive control groups (+ ctrl, mice injected with 2W1S in CFA) exhibited strong production of IFN- γ and IL-2 when stimulated with the immunizing peptide. Lymph node cultures from the positive control group showed production of IFN- γ but not IL-2 upon peptide stimulation (Figures 6 and 7). Notably, mice from the triblock group, which raised an antibody response against the triblock, did not appear to produce triblock-reactive T cells in their spleens or lymph nodes. This pattern of an antibody response with no associated splenocyte reactivity was also observed for epitope-displaying β -sheet fibrillizing peptides that we have previously investigated [21].

DISCUSSION

The main finding of this study was that the triblock peptide-PEG-peptide elicited a moderate antibody response, whereas the native peptide and γ KEI did not elicit any detectable response. None of the peptide groups elicited IL-2 or IFN- γ production in cultures of lymph node cells or splenocytes from immunized mice. The circular dichroism data indicated that both γ KEI and the triblock appeared to possess similar folding, but the triblock molecule was more highly oligomerized as observed by AUC. Collectively, these results suggested that the more extensive oligomerization of the triblock molecule led to the observed increase in its immunogenicity.

Supporting the interpretation that the triblock's oligomerization into higher molecular weight aggregates was the cause of the increased antibody production, other oligomerized peptide systems have shown similar effects. For example, immune responses to self-assembled β -sheet fibrillizing peptides displaying a known epitope on the fibrillar surface were similar to the immune responses observed for the triblock γ KEI-PEG- γ KEI molecule, though they appeared to be stronger for the β -sheet peptides [21]. For both the β -sheet fibril system investigated previously and the coiled coil triblock investigated in the present study, an antibody response was observed in the absence of any associated production of IFN- γ or IL-2 in splenocyte cultures [21]. In the β -sheet system, this response was not elicited by un-assembled epitope peptides without any adjuvant, nor was it elicited by mixtures of β -sheet peptide and soluble antigen peptides, indicating that immunogenicity was dependent on peptide assembly. Aggregation has also been shown to enhance the immunogenicity of recombinant protein therapeutics, and recent approaches in vaccine design have utilized supramolecular assembly to generate immunogens capable of inducing high antibody titers [20,50]. For some time it has been known that high molecular weight polymeric compounds with highly repetitive epitopes can activate T cell-independent (TI) antibody responses by delivering prolonged and persistent signaling to B cells [51]. Such TI antibody responses can be generated in the absence of any additional adjuvant [20,21,50]. Owing to the structural similarity between previously known TI antigens, the triblock investigated here, and the β -sheet fibrillar system reported previously, a similar type of response may have been elicited for the coiled coil triblock. The T cell independence of this response could be

definitively ascertained in future studies using T cell knockout mice or another model animal that cannot raise a specific T cell response.

The present study points to the oligomerization of the triblock being a factor that contributed to its immunogenicity, but other mechanisms cannot be explicitly ruled out. For example, although the folding between γ KEI and the triblock appeared to be highly similar by CD, there may have been subtle conformational changes between the two not detectable by this method. It is also not known if the native and γ KEI peptide would be immunogenic when delivered with an adjuvant like CFA, or if a native-PEG-native triblock could induce a response similar to γ KEI-PEG- γ KEI triblock. Investigating the immunogenicity of the peptides conjugated to linear, branched, or dendrimeric macromolecules could shed further light on the antigen valency necessary for producing the observed responses. In addition, the triblock possessed an additional Cys residue and a maleimide-thiol linkage to the PEG chain, possibly producing a novel linear epitope. In other contexts, maleimide linkages have been found to be capable of generating immune responses [52], and this possibility cannot be ruled out in the present system. Control peptides representing γ KEI functionalized with small maleimides would resolve this question. With regards to the ELISA measurements, their accuracy is supported by the fact that none of the peptides investigated had an abnormally high background when incubated with irrelevant sera. However, in almost all ELISAs, it is impossible to completely rule out the possibility that different samples could adsorb or present epitopes with different efficiencies on ELISA plates. Given the uniform background levels and the high-binding plates used, however, this seems unlikely.

The role of biomaterials as immune adjuvants is becoming increasingly appreciated. Pioneering work by Babensee's group has shown that biomaterials such as poly(lactic-co-glycolic acid) (PLGA), chitosan, hyaluronic acid, and agarose induce maturation of dendritic cells (DC) [53]. Immunizing mice with ovalbumin pre-adsorbed onto these polymeric biomaterials can induce moderate immune responses that can be sustained for as long as 18 weeks [54]. It has been hypothesized that such polymeric biomaterials induce DC activation in a manner analogous to pathogen associated molecular patterns (PAMPs), through pattern recognition receptors (PRRs) including Toll-like receptors (TLRs), or by contributing to the production of damage associated molecular patterns (DAMPs) at the tissue/biomaterial interface [53]. Dendritic cell maturation can be dependent on the physical shape and surface area of the implanted biomaterial [53], and antibody responses elicited by PLGA carriers have been shown to be primarily Th2 helper T cell-dependent, as illustrated by a predominance of IgG1 with respect to other antibody isotypes [54]. Although antibody isotypes were not investigated in the present study, previously investigated β -sheet fibrillizing peptides also exhibited strong IgG1 responses [21], indicating that there may be some similarities between the responses elicited by self-assembled peptide materials and those elicited by other biomaterials such as PLGA. Additionally, native ECM proteins including fibrinogen have been shown to activate macrophages and promote the attraction of immune infiltrates [55]. Although it seems unlikely, it is possible that the native immunogenicity of fibrinogen itself could contribute to the immune responses observed for the fibrin-based coiled coil materials described here.

The results of the experiments reported here have a few implications for the development of self-assembling biomaterials, particularly those that have a high degree of oligomerization or multivalency. Many biomaterials currently in development contain peptides, proteins, or other molecules in highly multivalent arrays. Ligand-functionalized polymer hydrogels and self-assembling biomaterials under development as cell scaffolds for regenerative medicine are particular examples [3-9,26]. The present study is part of a growing number of reports indicating that multivalency can lead to measurable antibody responses for such materials. The questions that remain open are how strong of an antibody response, and what type of an

antibody response, can be acceptable in different biomaterials contexts? Many applications of highly multimeric biomaterials with conjugated proteins or peptides have been investigated in vivo with good results [8,14,26,28,56], making it possible that some degree of antibody response may be acceptable or even desirable. It is expected that the acceptability of any antibody response would be highly dependent on the specific application and tissue site. Elucidation of the mechanisms of immune responses against multivalent materials will continue to be important considerations as they are developed further towards clinical use.

CONCLUSIONS

A series of peptides with variable abilities to form coiled coil bundles and oligomers was developed. A native peptide selected from mouse fibrin(ogen) did not form helical structures or self-assemble, but the engineered γ KEI peptide formed coiled coil bundles, and the triblock γ KEI-PEG- γ KEI formed coiled coil bundles and oligomers. In mice, neither the native peptide nor γ KEI elicited any detectable immune response, but the triblock molecule elicited a moderate antibody response. No production of IL-2 or IFN- γ was observed in cultures of challenged splenocytes from the immunized mice, with the exception of the positive control mice, which received peptide in adjuvant. Collectively, these results suggest that self-assembly and multimerization can influence the immunogenicity of peptide-based materials and that strategies for modulating these processes may benefit biomaterials development.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

This research was supported in part by the National Institutes of Health (NIDCR grant number R21DE017703 and NIBIB grant number 1R01EB009701). The content is solely the responsibility of the authors and does not necessarily represent the official views of the NIH. We thank Allyson Sholl and Adora Lin for help with the immunology studies and Stephen Macha for assistance with mass spectrometry. The circular dichroism and analytical ultracentrifugation studies were performed at the University of Chicago Biophysics Core facility.

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Figure 1.

Peptide sequences and schematics showing the native peptide from the gamma chain of mouse fibrin (γ 51-85) (a), the modified γ KEI peptide (b), and the peptide-PEG-peptide triblock (c). N-terminal helical wheel projections showing the residue positions for the native peptide (d), γ KEI (e, all substitutions are underlined and in bold), and a parallel coiled coil bundle of γ KEI illustrating an all-isoleucine core and charge complementarity between residues in *e* and *g* positions (f). ESI mass spectrometry of the triblock (g) showing characteristic 44 Da spacing of ethylene glycol units and an average m/z near the expected value of 12,144.

Figure 2. Circular dichroism of the native peptide (δ), γ KEI ($\circ$), and the triblock ($\square$) at concentrations of 20 μ M (a) and 40 μ M (b), in PBS, pH 7.4.

Figure 3. Sedimentation coefficient distributions for γ KEI peptide (dashed line) and the triblock (solid line) as determined by AUC sedimentation velocity. The γ KEI peptide sedimented as two discrete oligomers and did not form more highly oligomerized supramolecular structures. The triblock formed similar discrete bundles plus a significant amount of larger oligomers (inset).

Figure 4.

The native, γ KEI, and triblock peptides were non-cytotoxic to human umbilical vein endothelial cells by MTS assay. HUVECs were incubated with 1 mg/mL (white bars), 0.1 mg/mL (diagonally hatched bars), or 0.01 mg/mL (black bars) peptides in culture. Controls were culture media (grid), cultures with no peptide (horizontal bars) and killed cultures (diagonal). * $p < 0.05$ compared to cultures with no peptide by ANOVA using Tukey HSD post hoc test.

Figure 5. Antibody production in the serum of mice immunized with peptides and peptide-polymers. Each circle represents one mouse, and the bars represent the mean. * $p < 0.05$ compared to PBS, native, and engineered peptides by ANOVA with Tukey HSD post hoc test.

Figure 6.

IFN- γ production in cultures of spleen cells (a) and lymph node cells (b) from immunized mice. Hatched bars indicated cultures challenged with the immunizing peptide in vitro, and white bars indicate unchallenged cultures. * $p < 0.01$ compared to other groups by ANOVA with Tukey HSD post hoc test.

Figure 7. IL-2 production in cultures of spleen cells (a) and lymph node cells (b) from immunized mice. Hatched bars indicated cultures challenged with the immunizing peptide in vitro, and white bars indicate unchallenged cultures. * $p < 0.01$ compared to other groups by ANOVA with Tukey HSD post hoc test.

Table 1

Peptide helix positions (non-polar in bold), amino acid sequences, and substitutions (underlined).

Helix Position	<i>abcdefgabcdefgabcdefgabcdefgabcdefgab</i>
γ51-85 (native)	ADFLSSYQTDVDNDLRTLEDILFRAENRTTEAKEL
γKEI	<u>IDF</u> <u>I</u> SSY <u>I</u> TD <u>IDN</u> <u>K</u> IRT <u>I</u> ED <u>K</u> IF <u>E</u> ENR <u>I</u> TE <u>E</u> EL
Cys-γKEI	C- <u>IDF</u> <u>I</u> SSY <u>I</u> TD <u>IDN</u> <u>K</u> IRT <u>I</u> ED <u>K</u> IF <u>E</u> ENR <u>I</u> TE <u>E</u> EL
Human γKI [ref 13]	<u>IDF</u> <u>I</u> STY <u>I</u> TK <u>IDK</u> <u>K</u> IQS <u>I</u> EDI <u>I</u> HQ <u>I</u> ENK <u>I</u> SE <u>I</u> KQL <u>I</u> K