

RESEARCH ARTICLE

Immune-tolerant elastin-like polypeptides (iTEPs) and their application as CTL vaccine carriers

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Background: Cytotoxic T lymphocyte (CTL) vaccine carriers are known to enhance the efficacy of vaccines, but a search for more effective carriers is warranted. Elastin-like polypeptides (ELPs) have been examined for many medical applications but not as CTL vaccine carriers.

Purpose: We aimed to create immune tolerant ELPs using a new polypeptide engineering practice and create CTL vaccine carriers using the ELPs.

Results: Four sets of novel ELPs, termed immune-tolerant elastin-like polypeptide (iTEP) were generated according to the principles dictating humoral immunogenicity of polypeptides and phase transition property of ELPs. The iTEPs were non-immunogenic in mice. Their phase transition feature was confirmed through a turbidity assay. An iTEP nanoparticle (NP) was assembled from an amphiphilic iTEP copolymer plus a CTL peptide vaccine, SIINFEKL. The NP facilitated the presentation of the vaccine by dendritic cells (DCs) and enhanced vaccine-induced CTL responses.

Discussion: A new ELP design and development practice was established. The non-canonical motif and the immune tolerant nature of the iTEPs broaden our insights about ELPs. ELPs, for the first time, were successfully used as carriers for CTL vaccines.

Conclusion: It is feasible to concurrently engineer both immune-tolerant and functional peptide materials. ELPs are a promising type of CTL vaccine carriers.

Keywords

Cytotoxic T lymphocyte (CTL) vaccine, immune-tolerant elastin-like polypeptide, inverse phase transition, non-canonical elastin-like polypeptide motifs, reversible, vaccine carrier, thermally-induced

History

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Introduction

Vaccines that induce Cytotoxic T lymphocyte (CTL) responses are important prophylactic or therapeutic modules against cancer and infectious diseases [1–3]. Using carriers to promote these vaccines' delivery to antigen presenting cells is a strategy to enhance the potency of the vaccines [4,5]. While various natural and synthetic materials have been tested as building materials of CTL vaccine carriers [6,7], among them, only virus-like particles (VLPs) have been approved for clinical use to facilitate CTL responses [5], a contrast suggesting a need to further search for suitable vaccine carrier materials.

Elastin-like polypeptides (ELPs) share the same chemical nature, protein, with VLPs. ELPs can also self-assemble into nanoparticles (NPs) of a similar size as VLPs [8]. Besides these similarities to VLPs, ELPs have several additional features appealing to vaccine delivery. (1) The protein-or peptide-based CTL vaccines, potentially, can be fused

together with ELPs using genetic engineering approach; the resultant fusion proteins may be easily reproduced in *Escherichia coli* or other expression systems similar to some existing ELP fusions [9,10]. (2) When the vaccines are loaded to the carriers using the genetic engineering approach, the copy numbers of the vaccines and their cleavage sites from the carriers are well defined and precisely adjusted to improve the potency of vaccines [11–13]. (3) The signature property of ELP – the reversible, thermally (or ion)-induced, inverse phase transition – is transferable to ELP-protein fusions and possibly to iTEP-vaccine fusions [14]. The fusions can thus be simply purified by cycling the transition. Although having these appealing features, ELPs have not been reported as CTL vaccine carriers to date. We, thus, explored the possibility of using ELP NPs as CTL vaccine carriers in this study. There were evidences showing that humorally immunogenic carriers jeopardize the potency of their CTL vaccine payloads [15–17]. Therefore, we sought to use humorally tolerant ELPs as CTL vaccine carriers. Among reported ELPs, a few have been confirmed as immune tolerant while others have been proven immunogenic [18–23]. These immune tolerant ELPs, however, do not offer the required hydrophobicity and length to form NPs. This limitation prompted us to create new immune tolerant ELPs to meet the vaccine delivery need.

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Polypeptide materials, including ELPs, have typically been invented and optimized for physicochemical properties such as their phase transition property, and their immunogenicity was considered only after their physicochemical properties were established. Such practice, however, bears the risk that an ELP with a well-characterized function may indeed become valueless due to its later-discovered adverse immunogenicity [24,25]. Thus, we employed a new ELP development practice that places equal weight on ELP's phase transition feature and immunogenicity from the beginning of the development.

Using the new practice, we invented four sets of novel ELPs whose sequences happened to be non-canonical to ELPs' typical "V-P-G-X-G" motif [26,27]. We termed these novel ELPs as immune-tolerant elastin-like polypeptides (iTEPs) to underscore our new ELP engineering practice. We found all four sets of iTEPs possessed the desired transition property and were tolerated by mouse humoral immunity. We further paired two iTEPs that were opposite in hydrophobicity to make an amphiphilic diblock copolymer. The copolymer, when fused with a model CTL peptide vaccine, SIINFEKL, self-assembled into a NP. The NP enhanced the presentation of the vaccine by dendritic cells (DCs) and increased the strength of the vaccine-induced CTL response. Our results suggest that iTEPs developed with this new practice are suitable for CTL peptide vaccine carriers.

Method

Construction of iTEP expression plasmids

The genes encoding iTEPs were synthesized on a modified pET25b(+) vector using a reported method with some modifications [28]. First, a pET25b(+) vector was modified by inserting a double-stranded DNA at the vector's XbaI and BamHI endonuclease restriction sites. The inserted DNA was assembled by annealing together two complementary oligonucleotides, pET25-F and pET25-R (Table S1) (Eurofins Genomics, Huntsville, AL). The insertion of this DNA introduced two new restriction sites for BseRI and AcuI, and an in frame stop codon to the pET25b(+) vector. Second, genes that encoded subunits of iTEP_A: (GVLPGVG)₄, iTEP_B: (GAGVPG)₅, iTEP_C: (VPGFGAGAG)₃, and iTEP_D: (VPGLGAGAG)₃ were generated by annealing the sense and antisense oligonucleotides of these genes together (Table S1). Third, these iTEP genes were inserted to the modified pET25b(+) vector at its BseRI site. Finally, iTEP genes were extended to desired lengths through the following method. Specifically, the modified vector harboring iTEP genes were digested by two sets of enzymes, respectively. The first set includes AcuI, ApaI, and BglII; the second set includes BseRI and ApaI. Then, two DNA fragments that were from the two sets of digestions and contained iTEP genes were isolated and ligated together using T4 DNA ligase to create a new iTEP expression vector. The vector was transformed into DH5 α for its amplification. The lengths of iTEP genes which dictated repeat numbers of iTEP building blocks were confirmed by an XbaI and BamH I double digestion and the followed agarose gel analysis. This PRe-RDL process was repeated as desired to generate iTEP genes with designed lengths. The final iTEP genes were verified by DNA sequencing in combination with

an endonuclease digestion approach (Genewiz, South Plainfield, NJ).

The iTEPs used as representatives for this study were: iTEP_A having 28 repeats of GVLPGVG, iTEP_B having 70 repeats of GAGVPG, iTEP_C having 21 repeats of VPGFGAGAG, and iTEP_D having 96 repeats of VPGLGAGAG. iTEP_C which has an odd number of repeat was generated unexpectedly. iTEPs with the above lengths were used for this study primarily because they can be expressed from *E. coli* and their transition temperature, with or without salt, are between ambient temperature and 60 °C so that it is operationally possible to purify them by cycling the transition. In addition, these sizes of iTEP are in range of natural proteins so that we can study the immunogenicity of iTEPs as proteins instead of peptides.

The gene encoding an amphiphilic fusion, iTEP_B-iTEP_A, was generated by linking iTEP_B and iTEP_A genes together using the PRe-RDL method [28]. The gene encoding a fusion, iTEP_B-iTEP_A-pOVA was generated in a similar manner using a gene of pOVA (Table S1). It is noteworthy that pOVA has two copies of a CTL epitope, SIINFEKL [29]. There was one natural flanking residue on the both sides of SIINFEKL. The actual amino acid sequence of pOVA is ESIINFEKLTESIINFEKLT.

Production and purification of iTEPs and iTEP fusions

Competent BL21(DE3) *E. coli* cells (EMD Chemicals Inc., Gibbstown, NJ) were transformed with the pET25b(+) expression vector bearing iTEP or iTEP fusion genes. The single colony transformant was grown in TB media containing 100 μ g/mL ampicillin for 24 h at 37 °C. After the growth, *E. coli* cells were collected as a pellet by centrifugation for 25 min at 4816g and 4 °C. The cell pellet was then resuspended in phosphate buffered saline (PBS) and lysed by sonication for 3 min/L culture (sonication pulse rate: 10 s on and 30 s off). Later, 10% of polyethylenimine (PEI) was added to the cell lysate to precipitate *E. coli* DNA, and the precipitant was removed by centrifugation for 15 min at 21 000 g and 4 °C. Lastly, iTEPs or iTEP fusions were purified from the supernatant via inverse transition cycling (ITC) as described previously [30]. The purity of iTEP was assessed by SDS-PAGE using copper staining [31].

Endotoxin removal for iTEPs and iTEP fusions

Endotoxin inside of iTEPs or iTEP fusion samples was removed using Pierce High Capacity Endotoxin Removal Resin (Thermo Scientific, Waltham, MA) following the manufacturer's instruction. Residual endotoxin in the samples was determined by Limulus Amebocyte Lysate (LAL) PYROGENT Single Test Vials (Lonza, Allendale, NJ). All samples used for *in vitro* and *in vivo* immune assays had their endotoxin level below 0.25 EU per mg protein.

Characterization of thermally-induced, reversible, inverse phase transition of iTEPs and the iTEP_B-iTEP_A-pOVA fusion

The phase transitions of iTEP or iTEP fusions were characterized by turbidity changes of sample solutions as a

function of temperature. Specifically, the optical density at 350 nm (OD_{350}) of a sample solution was monitored using a UV-visible spectrophotometer equipped with a multi-cell thermoelectric temperature controller (Cary 300, Varian Instruments, Walnut Creek, CA) while the solution was heated from 20 °C to 80 °C and then cooled to 20 °C at a rate of 1 °C/min. The maximum first derivative of the turbidity curve of a sample was identified. The transition temperature (T_t) of the sample is the temperature that corresponds to the maximum derivative.

Characterization of the size of iTEP_B-pOVA and iTEP_B-iTEP_A-pOVA fusions

The particle size distribution of the iTEP fusions was determined by dynamic light scattering (DLS) using a Zetasizer Nano-ZS instrument (Malvern Instruments, Malvern, UK) as previously described [30]. The fusions were prepared at 5 μM and 25 μM in PBS and equilibrated at 37 °C for the measurement. The reported results represented the average particle size by number.

Negative-stain, transmission electron microscopy of the iTEP_B-iTEP_A-pOVA fusion

Small, 3.5 μL aliquots of assembled particles (50 μM) were applied to a continuous carbon support film (Ted Pella, Redding, CA). The sample was briefly washed with distilled water and then stained with 1% aqueous uranyl acetate. Micrographs were recorded on a DE-20 camera (Direct Electron, LP, San Diego, CA) equipped with a 5120 × 3840 Direct Detection Device (DDD[®]) sensor in a JEOL 2200FS microscope at a nominal magnification of 30 000 (1.72 Å per pixel) and at an acceleration voltage of 200 keV.

Immunization of iTEPs and collection of immune sera

All animal studies followed an approved protocol by the Institutional Animal Care and Use Committee (IACUC) at the University of Utah. C57BL/6 mice were immunized twice at their right hocks at a dose of 100 μg iTEPs per mouse. The two immunizations had a two week interval. At one week after the second immunization, 100 μL blood was collected from each immunized mouse. The blood samples were allowed to sit for 30 min to 1 h at room temperature to clot. Sera were collected from the blood samples after the samples were spun for 10 min at 14 000 rpm at 4 °C. The sera were kept at -80 °C before an analysis of the titers of iTEP-specific IgG.

Determination of IgG titers by ELISA

Ninety six-well ELISA plates were coated overnight at 4 °C with 100 μL/well of capture antigens [20 μg/ml corresponding iTEPs, ovalbumin (OVA), or mouse serum albumin (MSA)]. Plates were washed with PBS-0.02% Tween 20 (PBST) buffer and blocked with 200 μL/well of PBST buffer containing 1% BSA for 1 hour at room temperature. Mouse sera were serially diluted in the PBST-1% BSA buffer and added at 100 μL/well into the 96-well plate. The plates were incubated at 4 °C overnight. After thoroughly washing with PBST, 100 μL/well of 1 μg/mL detection antibody (horseradish peroxidase-

conjugated anti-mouse IgG) was added, and plates were incubated for 1 h at room temperature under continuous shaking. After washing with PBST, 100 μL/well of tetramethylbenzidine (TMB) substrate solution was added for 15–30 min with continual shaking. The reaction was stopped with 100 μL/well of 1 M H₂SO₄. Plates were read at OD 450 nm (-570 nm for wavelength correction).

The end point titer of a serum IgG was defined as the reciprocal of the higher serum dilution whose OD value from the ELISA assay is higher than a statistically valid cutoff. The titer results were expressed as IgG titers (Log₁₀) for each sample. The cutoff was established for individual ELISA assay that utilize a given capture antigen. So the cutoffs may be different for distinct capture antigens. Specifically, the cutoff was obtained by using PBS (negative control)-immunized serum to perform ELISA in the corresponding antigen-coated wells. The value of the cutoff was calculated using the following equation [32]:

$$\text{Cutoff} = \bar{X} + SD t\sqrt{1 + (1/n)}$$

where \bar{X} is the mean absorbance readings of independent PBS control sera, SD is the standard deviation of the readings, n is the number of independent PBS controls (mouse samples), t is the $(1 - \alpha)$ th percentile of the one-tailed t -distribution with $\nu = n - 1$ degrees of freedom.

Presentation of the CTL epitope, SIINFELK, by DCs

Cells of a murine DC line (DC2.4, a gift from K. Rock) [33] were plated at $2.5 \times 10^5/500$ μL/well in 24-well plates. About 500 μL of OVA, SIINFELK peptide, or iTEP_B-iTEP_A-pOVA NP (iTEP-pOVA NP hereafter) were dissolved in cell culture media and added to the wells containing DCs. The cells were further cultured for 16 h at 37 °C with 5% CO₂ before they were collected and washed with PBS. The MHC class I complex, H-2 k^b/SIINFELK presented on DC surface was stained with a PE-tagged monoclonal antibody 25-D1.16 (Biolegend, San Diego, CA, 1:100 dilution) and quantified with flow cytometry (5×10^4 events collected per sample). The data are presented as MFI normalized to the MFI of untreated DC2.4 cells.

B3Z CD8+ T cell hybridoma activation assay

B3Z cells (a gift from N. Shastri) are a CD8+ T-cell hybridoma engineered to secrete β-galactosidase when their T-cell receptors are engaged with an SIINFELK:H2K^b complex [34]. To do this assay, 1×10^5 DC2.4 cells/well were set in 96-well plates. OVA, SIINFELK peptide, and iTEP-pOVA NP at indicated concentrations were loaded into the DC culture for 16 h and then washed away. 1×10^5 B3Z cells/well were added to the DC2.4 cell culture and co-cultured with DC2.4 cells for 24 h. The cells were washed with PBS before 100 μL of lysis buffer (PBS with 100 mM 2-mercaptoethanol, 9 mM MgCl₂, 0.125% NP-40) together with 0.15 mM chlorophenol red β-galactoside substrate were added into the wells. After 4 h incubation at 37 °C, the reaction was stopped with 50 μL/well of 15 mM EDTA and 300 mM glycine. OD₅₇₀ of the reaction solutions was measured and was subtracted by their OD₆₃₀ values. The

subtracted OD₅₇₀ was used as an indicator as activation status of B3Z cells.

In vivo CTL response by ELISPOT IFN- γ assay

C57BL/6 mice were immunized subcutaneously with each immunogen (2 nmol SIINFEKL equivalents per mouse) together with incomplete Freund's Adjuvants (IFA; Sigma, St. Louis, MO) at their left flanks. The immunization was repeated at their right flanks one week later. At 10 days after the second immunization, mice were sacrificed, and the spleens were harvested. The spleens were teased into single-cell suspensions and filtered through nylon mesh (40 μ m). Red blood cells were lysed by Ammonium-Chloride-Potassium (ACK) lysing buffer. The washed and resuspended single cells were counted using Contess™ Automated Cell Counter (Invitrogen, Grand Island, NJ). Splenocytes (8×10^6 mL) were incubated in RPMI-1640 medium supplemented with 10% heat inactivated fetal calf serum, 2 mM glutamine, 100 units/mL penicillin and 100 μ g/mL streptomycin (Invitrogen, Grand Island, NJ), SIINFEKL peptide (2.5 μ g/mL) in a 14 mL polypropylene tissue culture tube for 48 h. The cells were then washed and recounted. 2×10^5 live cells in 100 μ L medium were loaded into wells of 96-well filtration plates (Millipore, Billerica, MA) coated with 5 μ g/mL of capture anti-mouse IFN- γ mAb (Clone: R4-6A2, Biolegend, San Diego, CA). Triplicates were set up for each condition. Cells were discarded after 24 h of culture and the wells were incubated with 2 μ g/mL of biotinylated, detection anti-mouse IFN- γ mAb overnight (Clone: XMG1.2-Biotin, Biolegend, San Diego, CA). After the unbound detection mAb were washed away from the wells, the bound mAb were detected using horseradish peroxidase (HRP Avidin, Biolegend, San Diego, CA) together with 3-amino-9-ethyl-carbazole (AEC) substrate (Sigma, St. Louis, MO). The membranes on the bottom of the wells were peeled off, and color spots on the membrane were scanned. The spots were automatically counted using ImageJ software.

Results

Design of iTEPs

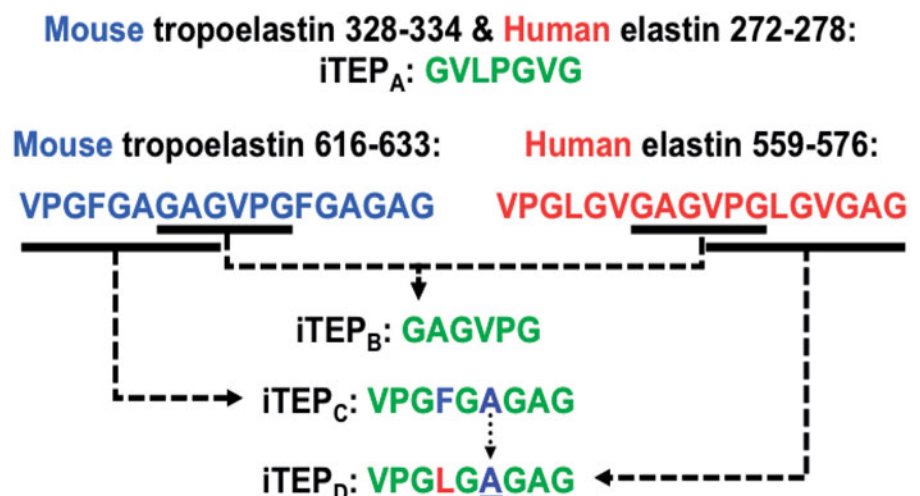
The iTEPs were designed as polymers of peptides derived from elastin. One critical criterion of these iTEPs is that they

should be humorally tolerant in both mice and humans, a feature that may facilitate their preclinical and clinical applications. For a polypeptide to be humorally immunogenic, it must contain at least one epitope to bind with B cell receptors (BCRs) and another epitope to bind first with the MHC class II complex, and then with a cognate T cell receptor (TCR) on CD4+ T cells [35–37]. By the same token, a humorally tolerant polypeptide should not contain TCR or BCR epitopes. In this study, homologous peptide sequences between human and mouse elastins were chosen as the monomers of iTEPs. These homologous sequences should intrinsically not bind with BCRs and TCRs of human and mouse; otherwise they would induce autoimmune responses (Figure 1).

Because iTEPs are polymers of elastin-derived peptides, the polymerization may introduce junction sequences that are exogenous to humans and mice, and these junction sequences are potentially humorally immunogenic. To diminish the possible immunogenicity, we utilized two strategies. First, we used the homologous peptides that repeat themselves twice in the elastins as monomers of iTEPs (see iTEP_C and iTEP_D, Figure 1), so there are no extrinsic junction sequences in these iTEPs. Further, the aforementioned, natural repeats have 18 residues and are longer than the typical length of MHC class II-restricted TCR epitopes (13–17 residues) and linear BCR epitopes (4–6 residue) [38,39]. Thus, the repeats are long enough to be used naturally to negatively select and deplete the BCRs and TCRs binding with them during lymphocyte development [40]. Consequently, no human and mouse BCRs or TCRs should bind with these repeats. iTEPs that are polymerized from these repeats, therefore, should be low- or non-immunogenic.

Our second strategy was applied when exogenous junction sequences were unavoidable. In this case, we had two criteria for monomers of iTEPs: one, they should be long homologous peptides from the elastins; two, they should be flanked by Gly at both ends (iTEP_A and iTEP_B, Figure 1). By using the longer monomers rather than shorter ones, we lowered the number of the junction sequences in iTEPs of a certain length. Potentially, this might lower immunogenicity of iTEPs, because the epitope density is a critical factor for the strength of immune responses [41–43]. The Gly-flanked monomers resulted in a high frequency of Gly inside the junction

Figure 1. An outline of iTEP monomer or dimers obtained from a homology analysis between mouse tropoelastin and human elastin. Online: Blue and red letters indicate residues from mouse and human elastins, respectively. Green letters indicate the same residues across both species. The numbers indicate the positions of these monomers or dimers in their parent elastin proteins.



sequences, which could mitigate the immunogenicity of the junction sequences as Gly had been shown to silence BCR epitopes [44,45]. With the second strategy, we expected iTEPs to have a low immunogenicity.

In order for our iTEPs to have the typical phase transition property of ELPs, all monomers of iTEPs were designed to contain only one proline and at least one valine (Figure 1). We hypothesized that this criterion is sufficient to render iTEPs the transition property, based on our studies of published ELP sequences [21]. It is noted that no iTEP monomers have the

canonical motif, VPGXG, of ELPs, which is not unexpected since the canonical motif was not a part of our iTEP design criteria.

Cloning and expression of iTEPs

iTEPs were produced and purified as recombinant proteins from *E. coli*. Their coding genes for iTEPs were constructed and elongated using a modified Pre-RDL method (Figure 2A) [28]. The coding genes were confirmed by DNA sequencing

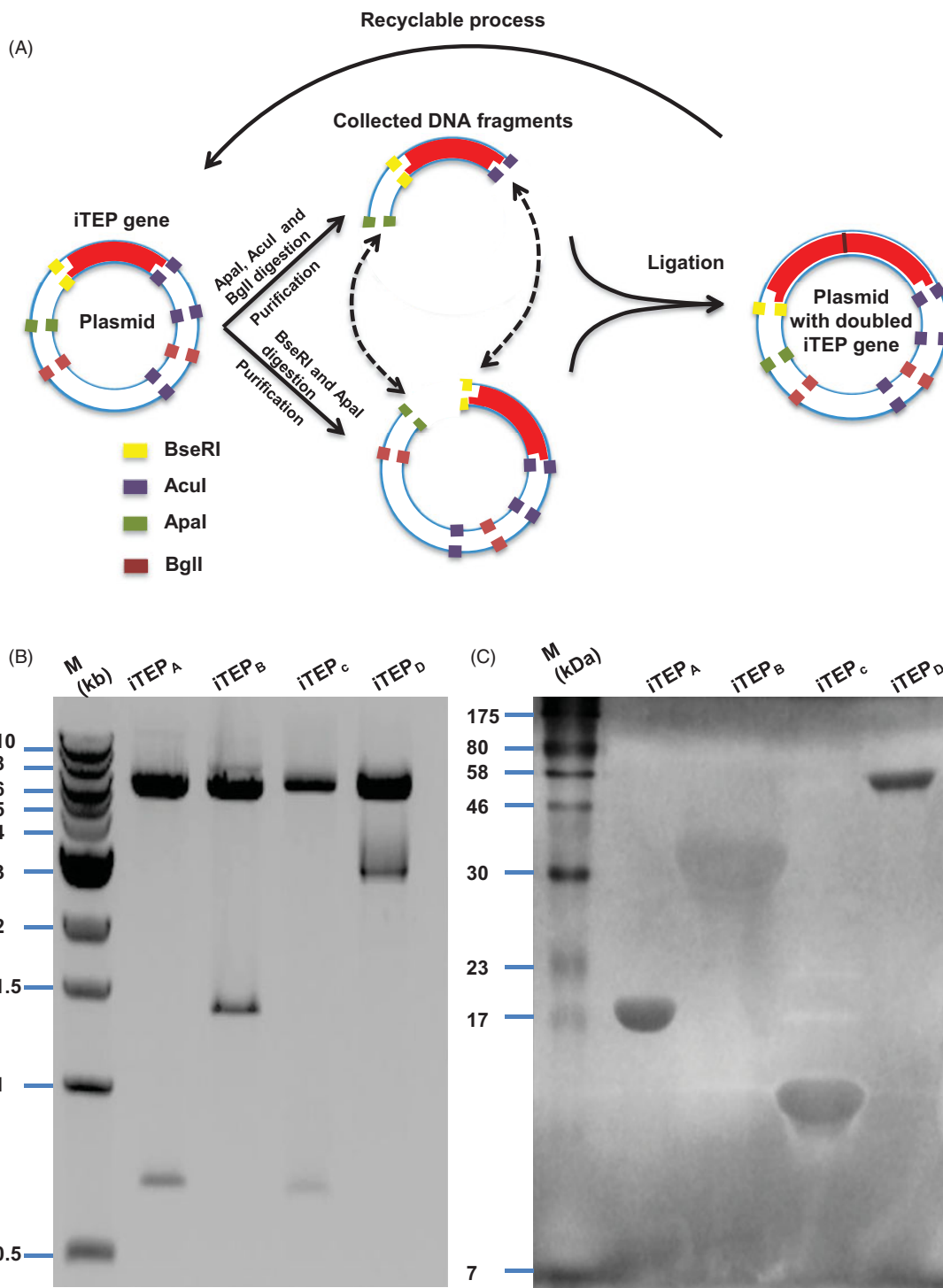


Figure 2. (A) Schematics showing the approach to double the length of iTEP coding genes. (B) iTEP coding genes on agarose gel after they were cleaved from pET25b(+) vectors by XbaI and BamHI. Sizes of these iTEP genes confirmed that these genes would code iTEPs of expected lengths. (C) SDS-PAGE gel showing MWs and purity of individual iTEPs.

in combination with endonuclease digestion approach. The agarose gel results (Figure 2B) confirmed the sizes of the coding genes: iTEP_A (600 bps), iTEP_B (1272 bps), iTEP_C (579 bps), and iTEP_D (2604 bps). Purity and sizes of the iTEPs after purification were confirmed by SDS-PAGE (Figure 2C). It is noteworthy that iTEP_B did not negatively stain as well as other iTEPs, probably, due to its hydrophilic yet uncharged nature (determined in the next section). The nature may hinder the emulsification of SDS around the iTEP, thus the iTEP can neither focus well on the gel nor prevent formation of copper complex at its migration band [46,47]. As a result, this iTEP appears as a faint smear on a copper-stained SDS-PAGE.

Thermally-induced, reversible phase transition of iTEPs

All four iTEP sets have variants displaying the phase transition feature (Figure 3A–E). The associated inverse transition temperatures (T_t) of these iTEPs are summarized together with their sequences and molecular weights in Table 1. iTEP_B displayed much higher T_ts than the other three iTEPs. Specifically, iTEP_B did not form coacervates in the tested temperature range (20–80 °C) in H₂O, while the others formed coacervates between 29 and 38 °C (Figure 3B versus

A, C, D). To achieve a thermally-induced phase transition of iTEP_B, we had to perform the experiment in a solution containing 2.5 M NaCl (Figure 3F). The high T_t of iTEP_B suggests it is much more hydrophilic than the other three iTEPs. Thus, among the four sets of iTEPs, we attained both hydrophilic and hydrophobic ones (Table 1).

An intriguing observation was made when T_ts of iTEP_C and iTEP_D (Figures 3C and D) were compared at two different concentrations, 5 and 25 μM. At 25 μM, iTEP_C had a lower (heating) T_t than iTEP_D, 35.7 °C versus 38.6 °C; at 5 μM, iTEP_C, however, displayed a higher T_t than iTEP_D, 50.5 °C versus 42.3 °C. The two iTEPs are similar to each other with only two differences: (1) the fourth residue of their repeat units, Phe for iTEP_C versus Leu for iTEP_D (Figure 1), and (2) the number of repeat units, 21 for iTEP_C versus 96 for iTEP_D (Table 1). While the first difference predisposes iTEP_C to a lower T_t than iTEP_D, the second difference predisposes iTEP_C to a higher T_t than iTEP_D [48,49]. At 25 μM, the impact of the fourth residue difference apparently prevailed over the impact of the repeat number difference; at 5 μM, vice versa. Thus, concentration changes of iTEPs apparently altered the impact of the two differentiating factors but at different scales. A clear delineation of this interesting observation will require a systematic study with two sets of iTEP_C and iTEP_D with comparable lengths.

Figure 3. (A–D) Turbidity profiles (OD₃₅₀) of iTEP_A, iTEP_B, iTEP_C, and iTEP_D as they were heated and then cooled between 20 °C and 80 °C in H₂O. (E) The turbidity profiles of iTEP_B in 2.5 M NaCl solution as a function of temperature. Each curve represents an average of three measurements.

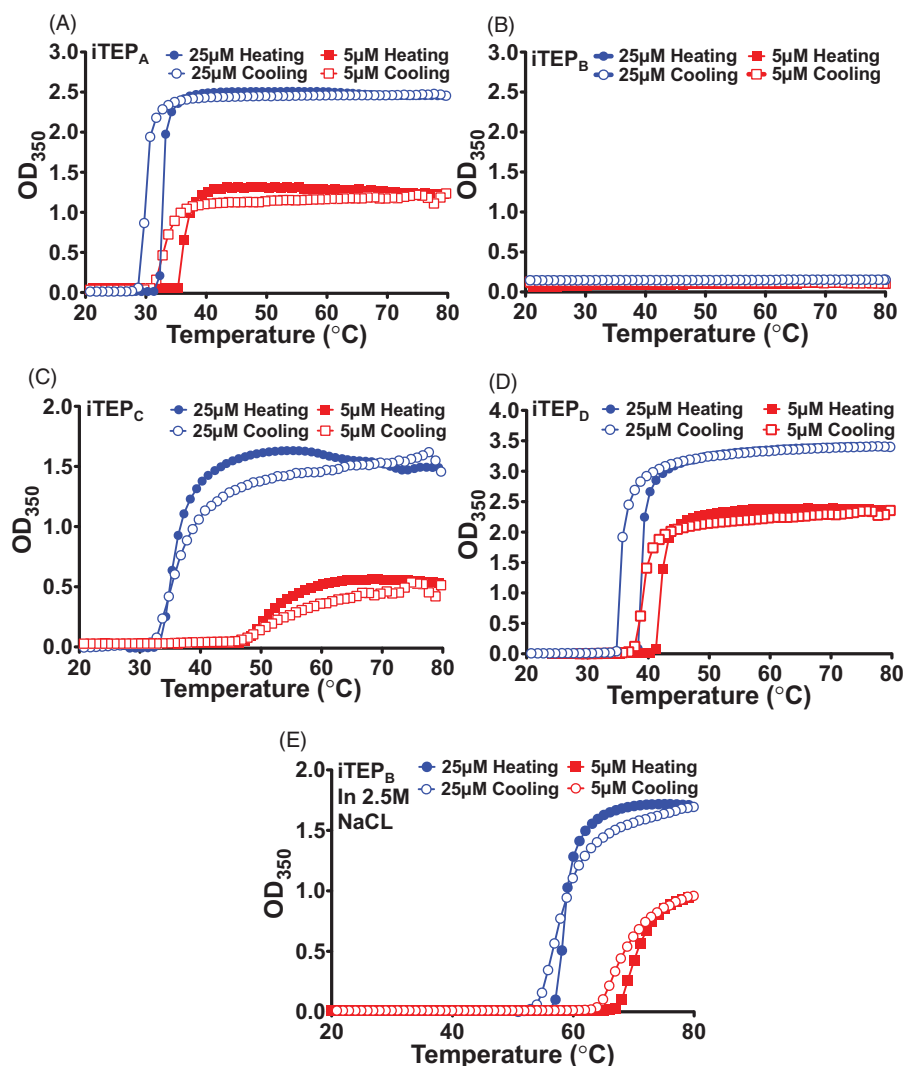


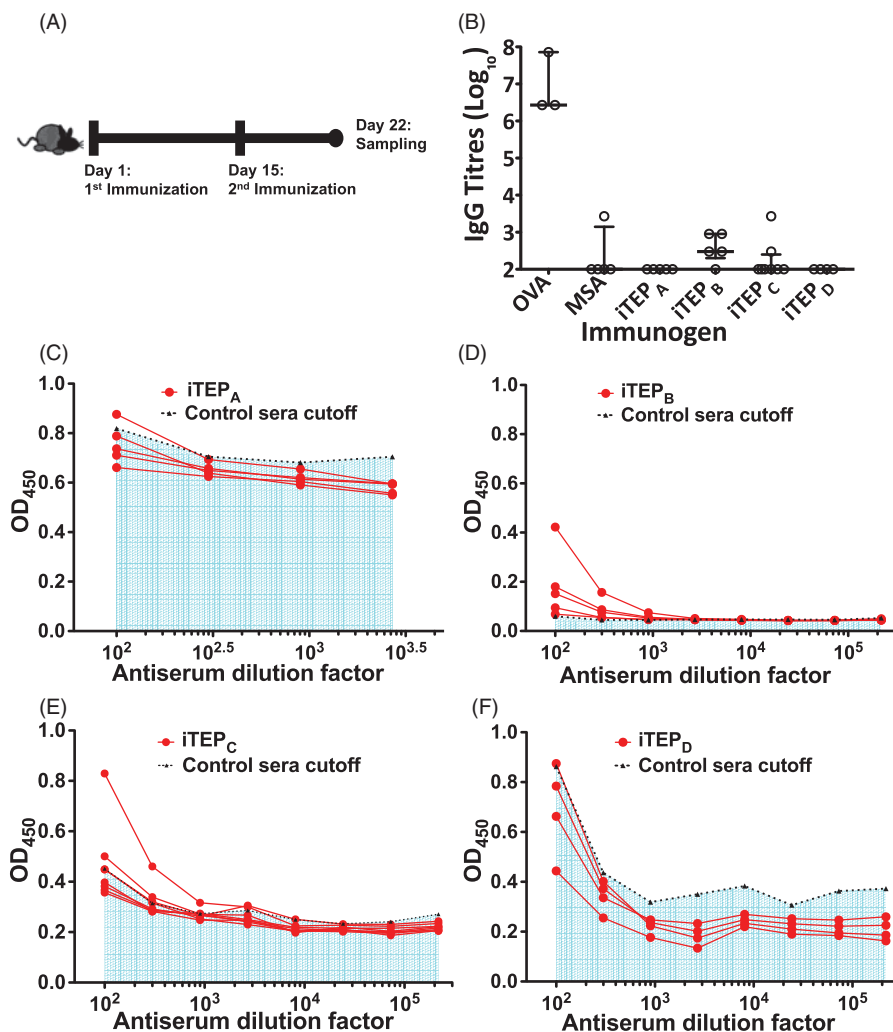
Table 1. The sequences, MW, transition temperatures of iTEPs.

iTEP	Construct sequence	MW (kDa)	Tt (heating) [°C]	Tt (cooling) [°C]	Relative hydrophobicity
iTEP _A	G(GVLPGVG) ₂₈ GG	16.6	33.30 ± 0.09	29.70 ± 0.05	High
iTEP _B	G(GAGVPG) ₇₀ GG	32.1	65.45* ± 0.15	60.85* ± 0.44	Low
iTEP _C	G(VPGFGAGAG) ₂₁ GG	15.3	35.70 ± 0.58	34.00 ± 0.58	High
iTEP _D	G(VPGLGAGAG) ₉₆ GG	65.6	38.60 ± 1.20	35.20 ± 0.57	High
iTEP _B -iTEP _A -pOVA	G(GAGVPG) ₇₀ G(GVLPGVG) ₂₈ G(ESIINFEKLT) ₂ GG	49.7	75.40 ± 0.14	73.70 ± 0.23	(N/A)

The Tt data presented in the table were obtained from experiments with sample's concentrations at 25 μM in H₂O except for iTEP_B.

*The Tts of iTEP_B was measured in the presence of 2.5 M NaCl.

Figure 4. (A) The immunization schedule and the time point of the assessment of humoral responses. (B) The summary of IgG titers of OVA, MSA, and iTEP-immunized mouse sera. Each dot represents titer result of one mouse serum. The medians and interquartile ranges of the titers are shown. (C–F) Absorbance (OD₄₅₀) of sera that were collected from iTEP_A (C), iTEP_B (D), iTEP_C (E), and iTEP_D (F) immunized mice after they were diluted and assayed by ELISA. Each data point corresponds to the mean value of three absorbance measurements of one serum dilution. The data of each mouse are linked together with a line. The cut-off ranges for positive absorbance values are shown as a blue shade.



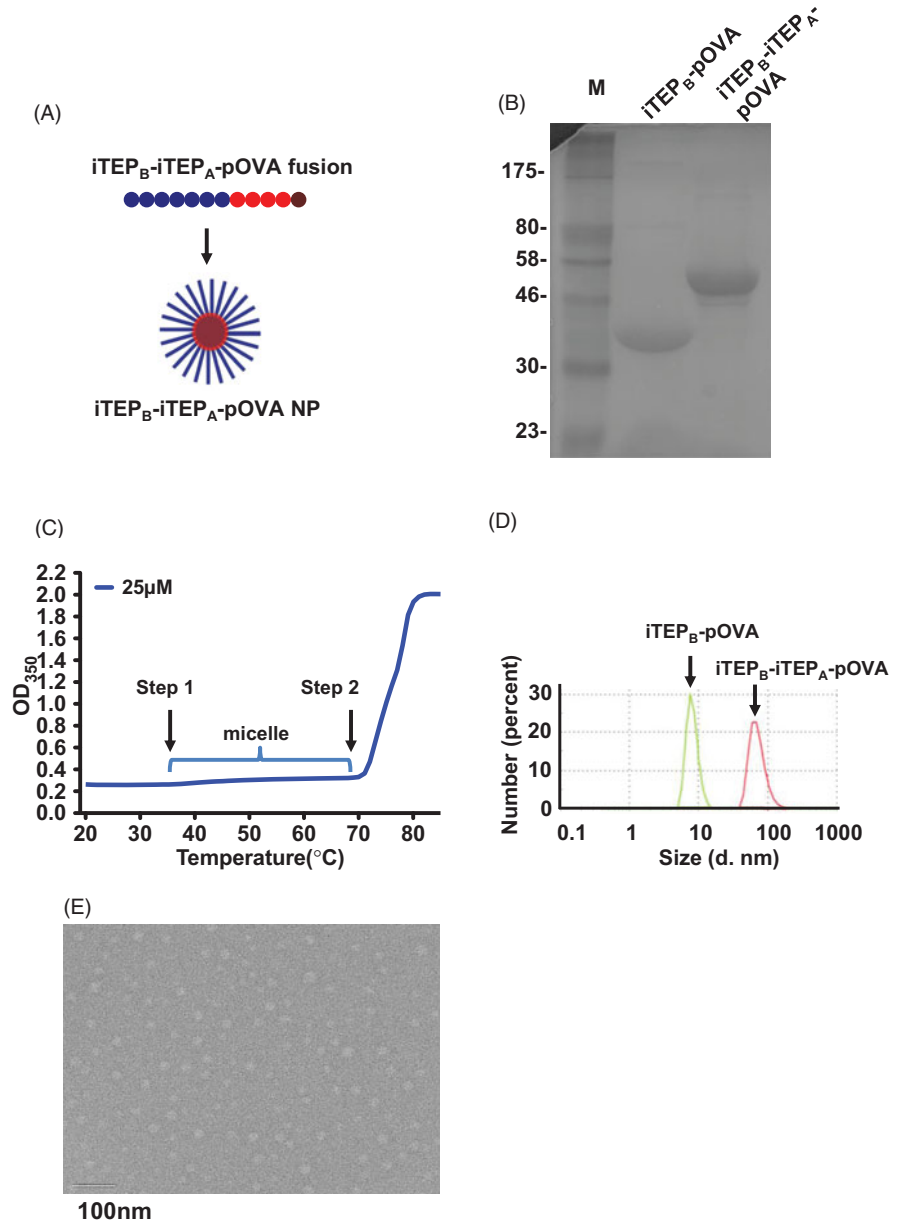
Humoral immunogenicity of iTEPs

Mice were immunized with the four iTEPs, a positive control (OVA), and a negative control (MSA) (Figure 4A). As expected, the OVA-immunized mice displayed a strong humoral response to OVA with a median OVA-specific antibody titer of 2.6×10^7 ; MSA-treated mice showed a very low humoral response, evidenced by a median MSA-specific antibody titer of 6.2×10^2 (Figure 4B). The sera of iTEP_A-, iTEP_C-, and iTEP_D-treated mice were negative for any iTEP-specific antibody after these sera were diluted by 100 times and up (Figure 4C, E, F), so their median titers should be less

than or equal to 100 (Figure 4B). The sera of iTEP_B-treated mice had a median antibody titer of 4.5×10^2 (Figure 4B and D). Because all of the iTEPs' titers are indifferent to that of MSA, but significantly different to the titer of OVA, we concluded that all iTEPs are humorally immune-tolerated by mice as MSA.

It has been reported that the aggregation of peptide and proteins could drastically increase their humoral immunogenicity [50,51]. However, aggregation status of the iTEPs did not appear to affect their immunogenicity. First, soluble and aggregate forms of iTEP_C did not show different immunogenicity (Figure S1). Second, all tested iTEPs are

Figure 5. (A) Schematics showing that the $iTEP_B$ - $iTEP_A$ -pOVA fusion self-assembles into a NP. (B) SDS-PAGE gel showing MWs and purity of two fusions, $iTEP_B$ -pOVA and $iTEP_B$ - $iTEP_A$ -pOVA. (C) Turbidity profile of the $iTEP_B$ - $iTEP_A$ -pOVA fusion (25 μ M) as a function of temperature. The solution has a slow, mild increase of turbidity since 33 $^{\circ}$ C (Step 1) and a sharp increase of the turbidity at about 70 $^{\circ}$ C (Step 2). The fusion assumes a micellar structure between these two temperatures. (D) Size distributions of the $iTEP_B$ -pOVA and $iTEP_B$ - $iTEP_A$ -pOVA fusions (25 μ M) obtained from DLS measurement. (E) A representative micrograph of negatively stained $iTEP_B$ - $iTEP_A$ -pOVA fusions confirming the NP size of the fusions.



non-immunogenic despite the fact that $iTEP_A$, $iTEP_C$, and $iTEP_D$ were injected as aggregates, while $iTEP_B$ was injected as a soluble molecule for the immunization.

NP self-assembled from $iTEP$ -CTL vaccine fusions

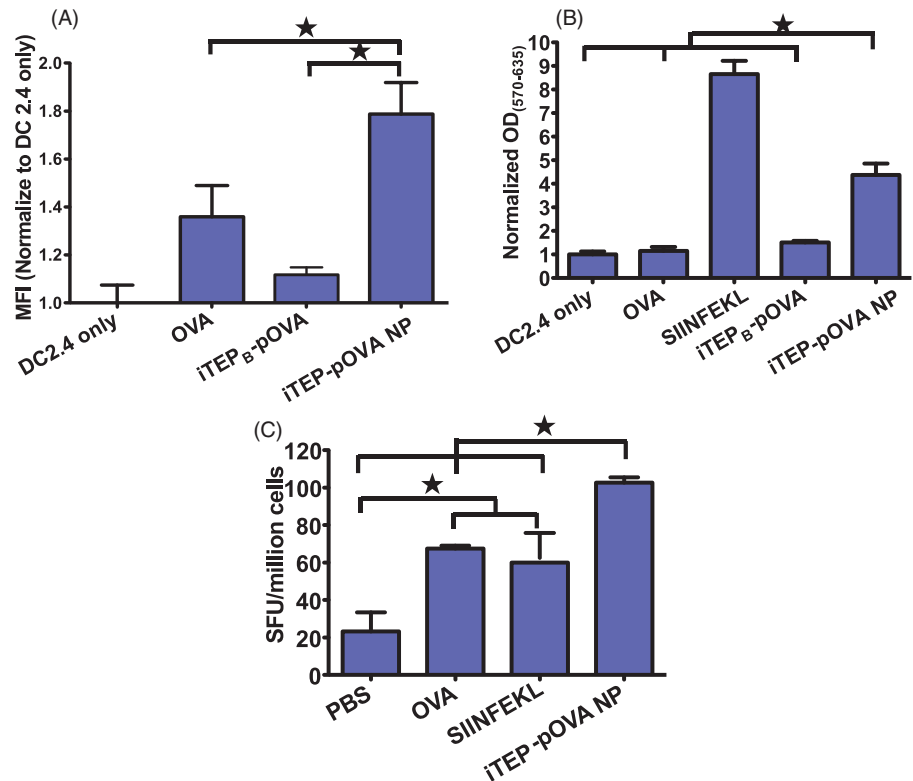
The hydrophobic $iTEP_A$, hydrophilic $iTEP_B$, and pOVA were fused together to form an amphiphilic $iTEP$ copolymer: $iTEP_B$ - $iTEP_A$ -pOVA (Figure 5A, Table 1). This pair of $iTEPs$ was used to construct the amphiphile because both the $iTEPs$ gave good yields and we expected that the amphiphile would have a good yield as well. Indeed, we were able to purify about 200 mg of amphiphilic fusion from one liter of culture (Figure 5B). The fusion displayed a micelle-like NP structure, evidenced by its two-step phase transition profile (Figure 5C) [8]. The NP structure of the fusion was also confirmed by DLS (Figure 5D), as well as electron microscopy (Figure 5E). According to DLS data, the fusion have a mean diameter of 81.2 ± 14.2 nm at 5 μ M and

71.9 ± 20.8 nm at 25 μ M (Figure 5D). Contrariwise, $iTEP_B$ -pOVA, a fusion of the hydrophilic $iTEP_B$ and the vaccine, is soluble and does not form NPs. The size of this fusion is less than 10 nm as measured by DLS (Figure 5D).

Immune responses induced by the $iTEP$ -pOVA NP

First, we tested whether the $iTEP$ -pOVA NP promotes surface presentation of SIINFEKL by DCs. The NP, soluble $iTEP_B$ -pOVA fusion, as well as OVA was incubated with DCs, respectively. The presentation of SIINFEKL by DCs was detected by an antibody that can recognize the SIINFEKL/H-2K^b complex. Although all of the above incubations led to the presentation, DCs incubated with the NP presented significantly more SIINFEKL epitopes than DCs with OVA or $iTEP_B$ -pOVA (Figure 6A). It is noteworthy that free SIINFEKL peptide resulted in much stronger a SIINFEKL presentation by DCs than the NP, OVA, or $iTEP_B$ -pOVA fusion (data not shown). The result was likely caused

Figure 6. (A) Presentation of SIINFEKL by DC 2.4 cells after the SIINFEKL was delivered by OVA, soluble iTEP_B-pOVA fusion, or iTEP-pOVA NP. Data are presented as means of normalized MFI \pm SD of the entire DC population used in the experiments ($n \geq$ four independent experiments). (B) Activation of B3Z cells after they were incubated with DCs that presented SIINFEKL. The DCs were pre-incubated with different forms of antigens as noted in the picture. Data are presented as mean \pm SD from three independent experiments). (C) *Ex vivo* analysis of active, SIINFEKL-restricted splenocytes cells from mice ($n = 3-5$) immunized with OVA, free SIINFEKL peptide, or iTEP-pOVA NP. The activation of the cells was characterized by using an IFN- γ -based ELISPOT assay. Data are presented as Spot Forming Units (SFU)/million cells \pm SD. For all panels, \star indicates $p < 0.05$, *t*-test between the paired treatments.



by a direct exchange between SIINFEKL with those epitopes that were originally presented on the DCs' surface. Thus, the presentation of free SIINFEKL peptide by DCs does not need an antigen processing by the cells. Consequently, the result of free SIINFEKL peptide is not comparable with the results of other forms SIINFEKL-containing antigens which require antigen processing before SIINFEKL is presented.

Next, we examined if the improved presentation of SIINFEKL by iTEP-pOVA NP-treated DCs can lead to a more efficient activation of SIINFEKL-restricted CD8 cells. We used B3Z cells as target cells to perform a CD8⁺ T cell activation assay. The B3Z cells that were co-cultured with the NP-pretreated DC2.4 cells were several fold more active than the B3Z cells co-cultured with the DC2.4 cells that were pretreated with other antigens. Specially, NP/DC-treated B3Z cells were 4.38, 3.81, or 2.9 fold more active than DC-treated B3Z cells, OVA/DC-treated B3Z cells, and iTEP_B-pOVA/DC-treated B3Z cells, respectively (Figure 6B). As a positive control, SIINFEKL peptide/DC treated B3Z cells showed the highest activity among all treatments (Figure 6B). This result is consistent with the observation that DCs much more efficiently presented SIINFEKL when the peptide was incubated with DCs as a free form.

Last, we examined whether the enhanced vaccine presentation and CTL activation caused by the iTEP-pOVA NP can translate into elevated CTL responses *in vivo*. To this end, we subcutaneously immunized C57BL/6 mice twice with PBS, OVA, SIINFEKL peptide, or the NP and collect splenocytes from these mice. We then quantified SIINFEKL-restricted, activated splenocytes using an IFN- γ -based ELISPOT assay. Splenocytes that release IFN- γ should be SIINFEKL-restricted CTLs. Both OVA and the free peptide immunization lead to a boost of the number of CTLs compared to the

PBS control. However, the NP-immunized mice had a much higher number of the CTLs (averagely 105 spots per million seeded splenocytes) than both OVA- and SIINFEKL peptide-immunized mice (averagely 61 and 60 spots per million seeded splenocytes, respectively) (Figure 6C). It is interesting that the free peptide did not induce the strongest SIINFEKL-specific CTL response *in vivo* even though it elicited the highest response *in vitro*, suggesting that the CTL peptide vaccines need a supportive carrier. The deficiency of the free peptide vaccine might be due to a fast clearance of the peptide after its immunization; thus the vaccine had very limited access to DCs and other antigen presenting cells.

Discussion

In this study, we took an initiative to test a new ELP design and engineering practice that emphasizes physicochemical properties as well as immunogenicity of the ELPs from the very beginning of ELP design. We designed and generated four families of novel ELPs, termed as iTEPs. These iTEPs not only are humorally tolerant but also possess a phase transition property. We further demonstrated that an amphiphilic iTEP copolymer self-assembles into a NP. The NP, when used to deliver a model CTL peptide vaccine, improved the potency of the vaccine in comparison to the vaccine delivered in free peptide or protein forms.

Adverse immunogenicity of the peptide and protein materials could compromise their functionality by blocking the materials' interactions with their targets, shortening their half-lives [52,53], and decreasing their bioavailability [54]. Immunogenicity can also be life-threatening [54]. In the case of CTL vaccine delivery, it was suggested that humoral responses against vaccine carriers impede the effectiveness

of their vaccine payloads [55,56]. Since our motivation in this study is to explore ELPs' potential as CTL vaccine carriers, we started the study by creating iTEPs so that immunogenicity of these polypeptides would not be a hurdle for their usage as the carriers. The need of creating iTEPs was substantiated by a very recent report showing that ELPs having distinct sequences displayed very different humoral immunogenicity when they were complexed with plasmid DNAs [22]. To create iTEPs, we took an unconventional polypeptide design and engineering practice that incorporates both functionality and immunogenicity criteria in the design. This practice was to minimize the risk that a material with a well characterized function may become valueless due to its afterward discovered adverse immunogenicity. This practice, indeed, has proven fruitful to our purpose. We have generated four sets of iTEPs which have desired low immunogenicity.

Besides as a proof-of-principle for the new ELP development practice, the success of generating these iTEPs has a practical significance. These iTEPs are unique and likely more valuable in preclinical and clinical applications than other reported ELPs because they were designed as immune tolerant materials to both mice and humans, which is unprecedented [18–21]. Currently, iTEPs' immune tolerance in mice has been confirmed. Because the underlying ideas to render the iTEPs immune tolerated by mice and humans are the same, we believe that these iTEPs will be tolerated by human immunity when they are tested.

One of our overarching goals is to apply our new practice to the development of immune tolerant or immunogenic polypeptide materials beyond ELPs. While this goal can only be fully achieved after a broader investigation using additional model polypeptides, our results to some extent affirmed our criteria of designing non-immunogenic polypeptides. It is interesting that the iTEPs tested were found non-immunogenic regardless of their aggregation status or their nature as repetitive sequences, two auxiliary factors that were reported to possibly boost immunogenicity of polypeptides and proteins [51,57,58]. The result is consistent with the notion that iTEPs likely do not contain BCR or TCR epitopes, or both. If a polypeptide doesn't consist of BCR or TCR epitopes, it should not induce humoral responses even when those auxiliary factors favor such. More significantly, because two different strategies were utilized to diminish potential immunogenicity of iTEPs' junction sequences, our results regarding the immunogenicity of iTEPs also suggest that both strategies are valid and will likely be useful to generate other immune tolerant polypeptides. To further establish our strategy to design polypeptide materials with pinpointed immunogenicity, we are employing the same strategy in an opposite way to generate immunogenic ELPs.

The iTEPs discovered in this study possess the characteristic phase transition of ELPs without having their typical VPGXG motif. On the one hand, it is reasonable because we did not limit iTEP design to the motif. We reasoned that generating an immune tolerant ELP for both humans and mice is more important to the goal of the study than generating an ELP fitting in with the motif, although the two needs are not necessarily mutually exclusive. On the other

hand, this result may lead to a shift of the paradigm on the relationship between the VPGXG motif and the phase transition [59]. The fact that we were able to invent new ELPs outside of the conventional VPGXG motif offers us a greater freedom and power to create novel and functional ELPs in the future.

ELPs have been widely tested in biomedical applications [27,60–67]. However, they have not been used as CTL vaccine carriers although they can assemble into nanostructures that might be useful for vaccine delivery [9,62]. Here, we showed that an iTEP NP actually boosted the potency of the vaccines it delivered. This result suggests a new horizon for utilizing ELPs in vaccines and immunotherapy. This could be further substantiated if we can pinpoint immunogenicity of ELPs depending on their potential applications. For example, if we can generate both immunogenic and nonimmunogenic ELPs and their corresponding carriers, we could use this pair of otherwise very similar carriers to elucidate how the immunogenicity of carriers affect the potency of their vaccines or other immunotherapeutics payloads, which has not been clarified so far. In summary, an ability to precisely control the immunogenicity and functionality of ELPs is important to use the materials in delivering immunotherapeutics, which is still at its infancy [23].

Conclusion

We have successfully created four sets of non-canonical ELPs (iTEPs) that possess the inverse phase transition property and are immune-tolerated by mice. The success validates our mechanistic understanding about the phase transition and immunogenicity of ELPs. These iTEPs may be used for many reported biomedical applications of ELPs and possess an advantage for being non-immunogenic. Importantly, our study also demonstrated, for the first time, that ELPs can be used as CTL vaccine carriers, a very important but previously untested medical application. Most significantly, our novel polypeptide development practice, which places an equal design emphasis on functionality and immunogenicity, will help to avoid squandering efforts on developing functional ELPs that turn out to be immunogenic.

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Declaration of interest

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Supplementary material available online
Supplementary Figure S1 and Table S1