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Effect of protein immunogenicity and PEG size and branching on the anti-PEG immune response to PEGylated proteins

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ABSTRACT

PEGylation has successfully improved the pharmacological properties of therapeutic proteins. However, polyethylene glycol (PEG) has been burdened by immunogenicity that renders a negative clinical effect on therapeutic proteins. The anti-PEG immune response to PEGylated proteins possibly depends on the nature of proteins and the conjugated methoxy PEG (mPEG). Thus, it is necessary to investigate the effects of protein immunogenicity, the extent of PEGylation, the molecular weight (Mw), and the branching of mPEG on the anti-PEG immune response. Ovalbumin, tetanus toxoid (TT), TT–TT conjugate, and TT–bovine serum albumin conjugate were used as target proteins. PEGylated proteins with different extents of PEGylation were obtained by fractionation of the PEGylated TT with size exclusion chromatography. The PEGylated proteins with different Mw and branching of mPEG were obtained by modification of TT with linear mPEG (5 kDa and 20 kDa) and branched mPEG (20 kDa). The PEGylated proteins elicited high levels of anti-PEG antibodies (predominantly IgM and IgG1). The anti-PEG immune response depended on the immunogenicity of proteins, the extent of PEGylation, and the Mw of mPEG. In contrast, branching of mPEG had an insignificant effect on the anti-PEG immune response to the PEGylated proteins.

1. Introduction

Polyethylene glycol (PEG) has been successfully used to improve the pharmacological and biological properties of therapeutic proteins [1]. PEGylation prolongs the circulation time of proteins by enhancing their hydrodynamic radii and decreasing the kidney filtration [2]. PEGylation also increases the stability of proteins and decreases the immunogenicity of proteins [3]. Recently, several PEGylated proteins have been approved for clinical use, including Oncaspar (PEG-asparaginase, Ovation, USA) and Krystexxa (PEG–uricase, Savient, USA) [4]. These PEGylated proteins show higher therapeutic efficacy than non-PEGylated counterparts. Thus, PEGylation of proteins has been considered as a milestone breakthrough in the field of therapeutic proteins [5].

PEG has very low toxicity and its simple structure is assumed to be weakly immunogenic. However, the anti-PEG immune response has burdened the development of PEGylated proteins [6,7]. For instance, Oncaspar and Krystexxa can induce high level of anti-PEG antibodies and lead to unexpected immune-mediated side effects [8,9]. Anti-PEG antibodies have also been reported to accelerate the clearance and reduce the efficacy of PEGylated proteins [10,11].

The immune response against the PEG portion of a PEGylated protein was first reported in 1983 [12]. In brief, anti-PEG antibodies in rabbits were raised by immunization with PEGylated ovalbumin (OVA), PEGylated bovine superoxide dismutase, and PEGylated ragweed pollen extract in Freund's complete adjuvant (FCA). In contrast, the PEGylated proteins elicited weak or undetectable level of anti-PEG antibodies in the absence of FCA [12]. The authors concluded that the anti-PEG immune response depended on the nature of proteins and the degree of modification. however, this conclusion may be interfered by the presence of FCA and needs further demonstration. Sherman et al. assessed the role of methoxy group in the immune responses to methoxy PEG (mPEG) conjugates and the potential advantages of replacing mPEG with hydroxyl–PEG [13]. Saifer et al. found that the clinical use of hydroxyl–PEG–protein conjugates could induce less intense anti-PEG immune responses than the use of mPEG–protein conjugates [14]. Cheng et al. reported that anti-PEG IgM accelerated the clearance of PEGylated proteins [10]. Mima et al. reported that anti-PEG IgM was a major contributor to the accelerated blood clearance of PEGylated proteins [11].

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Anti-PEG antibodies are also implicated in the increased clearance of PEGylated liposomes after repeated administration in animal studies [15]. Several studies have shown that anti-PEG antibodies (predominant IgM) are associated with the rapid clearance of subsequent doses of PEGylated liposomes [16]. PEGylated liposomes can activate the complement system and cause hypersensitivity reactions [15,17]. Extensive studies suggest that anti-PEG IgM is secreted by splenic B cells without the stimulation of T helper cells and the immune memory is thus not induced [18]. This is only the case for PEGylated liposomes, but not for PEGylated proteins.

Anti-PEG immune response to the PEGylated proteins possibly depends on the nature of proteins and the conjugated mPEG. Thus, it is necessary to investigate the effects of protein immunogenicity, the extent of PEGylation, the molecular weight (Mw), and the branching of mPEG on the anti-PEG immune response. In addition, PEGylated proteins with strong anti-PEG immunogenicity are highly preferred to immunize the animals in the absence of adjuvants.

In the present study, OVA, tetanus toxoid (TT), TT–TT conjugate (TT–TT), and TT–bovine serum albumin conjugate (TT–BSA) were of different immunogenicities and acted as target proteins. The PEGylated proteins with different PEGylation extents were obtained by fractionation of the PEGylated TT, using size exclusion chromatography (SEC). The PEGylated proteins with different Mw and branching of mPEG were obtained by modification of TT with linear mPEG (5 kDa and 20 kDa) and branched mPEG (20 kDa). The PEGylated proteins were used to immunize the BALB/c mice in the absence of adjuvants. The immunological characteristics of the PEGylated proteins were then investigated.

2. Materials and methods

2.1. Materials

Horseradish peroxidase-conjugated goat anti-mouse IgG Fc antibody (HRP-anti-IgG), IgG1 Fc antibody (HRP-anti-IgG1), IgG2a Fc antibody (HRP-anti-IgG2a), IgG2b Fc antibody (HRP-anti-IgG2b), IgG3 Fc antibody (HRP-anti-IgG3), and IgM Fc antibody (HRP-anti-IgM) were purchased from Abcam (USA). Bovine serum albumin (BSA), OVA, 3-maleimidohexanoic acid *N*-hydroxysuccinimide ester (EMCS), *N*-ethylmaleimide (NEM), 3,3′,5,5′-tetramethylbenzidine (TMB), 5,5′-dithio-bis-(2-nitrobenzoic acid), and 2-iminothiolane (IT) were purchased from Sigma (USA). Linear mPEG maleimide with Mw of 5 kDa (L–PEG5K–mal) and linear mPEG succinimidyl carbonate with Mw of 5 kDa (L–PEG5K–sc) were ordered from Jenkem Biotech (China). Linear mPEG maleimide with Mw of 20 kDa (L–PEG20K–mal) and branched mPEG maleimide with Mw of 20 kDa (B–PEG20K–mal) were purchased from Sinopeg Biotech (China). TT was kindly provided by Hualan Biological Engineering, Inc. (China).

2.2. Preparation and purification of TT–BSA

TT–BSA was prepared as described previously [19]. In brief, TT (8 mg/ml, 1.5 ml) was incubated with 80-fold molar excess of IT in 20 mM phosphate buffer ((PB) pH 7.2) at 4 °C for 3 h, followed by removal of the free IT with extensive dialysis against PB. Then, BSA (4 mg/ml, 1.5 ml) was incubated with 40-fold molar excess of EMCS in PB at 4 °C overnight, followed by removal of the free EMCS with extensive dialysis against PB. Finally, TT–BSA was prepared by incubation of EMCS–modified BSA (3 mg/ml, 1.5 ml) with the thiolated TT (6 mg/ml, 1.5 ml) at 4 °C overnight.

TT–BSA was purified from the reaction mixtures by SEC, using a Superdex 200 column (2.6 cm × 60 cm, GE Healthcare, USA). The column was equilibrated and eluted with PB at a flow rate of 2.0 ml/min. The effluent was detected at 280 nm. TT–BSA was fractionated and concentrated by centrifugation at 6000*g*, using a Centricon with a 50–kDa cutoff membrane (Millipore, USA). The concentrated sample was stored at −80 °C.

2.3. Preparation and purification of the TT–TT conjugate

TT (8 mg/ml, 1.5 ml) was incubated with 40-fold molar excess of EMCS in PB at 4 °C overnight, followed by removal of the free EMCS with extensive dialysis against PB. The conjugate (TT–TT) was prepared by incubation of EMCS–modified TT (6 mg/ml, 1.5 ml) with the thiolated TT (6 mg/ml, 1.5 ml) in PB at 4 °C overnight. TT–TT was purified from the reaction mixtures by SEC, using a Superdex 200 column (2.6 cm × 60 cm). The column was equilibrated and eluted with PB at a flow rate of 2.0 ml/min. The effluent was detected at 280 nm. TT–TT was fractionated and concentrated by centrifugation at 6000*g*, using a Centricon with a 50–kDa cutoff membrane (Millipore, USA). The concentrated sample was stored at −80 °C.

2.4. Preparation and purification of TT–L–P5K

The thiolated TT (6 mg/ml, 1.5 ml) was incubated with 80-fold molar excess of L–PEG5K–mal in PB at 4 °C overnight. The residual thiols of TT were blocked by NEM. The reaction mixture was loaded on a Superdex 200 column (2.6 cm × 60 cm). The column was equilibrated and eluted with PB at a flow rate of 2.0 ml/min. TT–L–P5K–Hi and TT–L–P5K–Lo were fractionated, respectively. The fractions were centrifuged and concentrated at 6000*g* by a Centricon, using a 50–kDa cutoff membrane. The concentrated sample was stored at −80 °C.

2.5. Preparation and purification of TT–L–P20 K and TT–B–P20K

The thiolated TT (6 mg/ml, 1.5 ml) was incubated with 60–fold molar excess of L–PEG20K–mal and 60–fold molar excess of B–PEG20K–mal in PB at 4 °C overnight, respectively. The residual thiols of TT were blocked by NEM. TT–L–P20 K and TT–B–P20 K were both subjected to a tangential flow filtration, using a 100–kDa cutoff membrane to remove the free PEG.

2.6. Preparation and purification of OVA–P5K

OVA (4 mg/ml, 1.5 ml) was incubated with 40-fold molar excess of IT in PB at 4 °C for 3 h, followed by removal of the free IT with extensive dialysis against PB. Then, the thiolated OVA (3 mg/ml, 1.5 ml) was incubated with 60–fold molar excess of L–PEG5K–mal in PB at 4 °C overnight. OVA–P5 K was subjected to a tangential flow filtration, using a 50–kDa cutoff membrane to remove the free PEG.

2.7. Preparation and purification of hGH–P5K

Human growth hormone (hGH) was expressed and purified as described previously [20]. hGH (4 mg/ml, 1.5 ml) was incubated with 40–fold molar excess of L–PEG5K–sc in PB at 4 °C overnight. hGH–P5 K was subjected to a tangential flow filtration, using a 50–kDa cutoff membrane to remove the free PEG.

2.8. Preparation and purification of TT–BSA–P5K

TT–BSA (6 mg/ml, 1.5 ml) was incubated with 60–fold molar excess of L–PEG5K–mal in PB at 4 °C overnight. The residual thiols of TT–BSA were blocked by NEM. TT–BSA–P5 K was subjected to a tangential flow filtration, using a 50–kDa cutoff membrane to remove the free PEG.

2.9. Preparation and purification of TT–TT–P5K

TT–TT (6 mg/ml, 1.5 ml) was incubated with 60–fold molar excess of L–PEG5K–mal in PB at 4 °C overnight. The residual thiols of TT–TT were blocked by NEM. TT–TT-P5 K was subjected to a tangential flow filtration, using a 50–kDa cutoff membrane to remove the free PEG.

2.10. SEC analysis

SEC analysis was carried out on an analytical Superdex 200 column (1 cm × 30 cm) at room temperature. The column was equilibrated and eluted by PB at a flow rate of 0.5 ml/min. The effluent was monitored at 280 nm.

2.11. Dynamic light scattering

The molecular radii of the samples were measured by dynamic light scattering (DLS) on a Zetasizer nano S photometer (Malvern Instruments, Malvern, UK) at 25 °C. The depicted data represented the average mean intensity from the volume particle size distribution of 16 measurements for each sample. All the samples were at a protein concentration of 1.0 mg/ml in PB. The samples were centrifuged at 12,000*g* for 10 min before analysis.

2.12. Circular dichroism spectroscopy

The far-UV circular dichroism (CD) spectra of the samples were recorded on a Jasco-810 spectropolarimeter (Jasco, Japan) between 260 nm and 190 nm. A cuvette with a 0.2 cm path length was used. All the samples were at a protein concentration of 0.1 mg/ml in PB. The buffer baseline was subtracted from the experimental spectra for corrections.

2.13. Quantitative assay

Bicinchoninic acid protein assay kit (Vigorous Biotechnology, Beijing, China) was used to measure the protein concentrations of the PEGylated proteins, using BSA as the standard [21]. The thiol levels in the thiolated proteins were measured using 5,5′-dithiobis-(2-nitrobenzoic acid) [22]. The extents of PEGylation in the PEGylated proteins were calculated using the difference of the thiol group levels between the thiolated proteins and the PEGylated proteins. The amine levels of hGH and hGH–P5K were measured by 2,4,6-trinitrobenzenesulfonic acid (TNBS) assay [23]. The number of amino groups of hGH available for PEGylation was ~7.6, as revealed by the TNBS assay. The extent of PEGylation in hGH–P5K was calculated using the difference in the amine levels between hGH and hGH–P5K.

A Superdex 200 column (1 cm × 30 cm) was used to measure the Mw of TT–BSA. A series of protein standards (carbonic anhydrase, BSA, alcohol dehydrogenase, β–amylase, apoferritin, and thyroglobulin) were used to calibrate the column. The Mw of TT–BSA was calculated to be ~289.0 kDa, using the standard curve. Thus, TT was conjugated with an average of ~2.1 BSA molecules in TT–BSA provided by the other measurement of heterogeneity. The number of conjugated BSA molecules was calculated to be an average of ~2.0 by comparing the difference in the thiol group levels between the thiolated TT and TT–BSA, using 5,5′-dithio-bis-(2-nitrobenzoic acid) [22]. Thus, the number of ~2.0 was used to calculate the TT content of TT–BSA. The extents of PEGylation in the other PEGylated proteins were calculated using the difference in the thiol group levels between the thiolated proteins and the PEGylated proteins.

2.14. Immunization

BALB/c female mice of weight 15–20 g were supplied by the Animal Center of Peking University Health Science Center (Beijing, China). All procedures of the animal experiments were approved by the Animal Ethical Experimentation Committee of Institute of Process Engineering, Chinese Academy of Sciences (Beijing, China), according to the requirements of the National Act on the Use of Experimental Animals (China).

Fifty-four mice were randomly divided into nine groups of six animals each. The groups were immunized with TT, TT–TT, TT–BSA, TT–BSA–P5 K, TT–TT-P5 K, TT–L–P5K–Hi, TT–L–P5K–Lo, OVA, and OVA–P5 K. The groups were defined according to the immunized samples. The samples were filtered with a 0.22–μm membrane before immunization. The mice were immunized intraperitoneally with the corresponding samples (0.1 ml) at a protein concentration of 0.2 mg/ml on days 0, 7, 14, 21, and 28, respectively. Blood samples were collected from the mice on days 7, 21, and 35. The mice sera were isolated and stored at −80 °C until use.

Twenty-four mice were randomly divided into four groups of six animals each. The groups were immunized with TT, TT–L–P5K–Lo, TT–L–P20 K, and TT–B–P20 K, respectively. The groups were defined according to the immunized samples. The samples were filtered with a 0.22–μm membrane before immunization. The mice were immunized intraperitoneally with the corresponding samples (0.1 ml) at a TT concentration of 0.3 mg/ml on days 0, 7, 14, 21, and 28. Blood samples were collected from the mice on days 14 and 35. The mice sera were isolated and stored at −80 °C until use.

2.15. Determination of antibodies

Anti–PEG, anti–TT, and anti–OVA antibodies in the mouse sera were measured using 96–well ELISA plates (Corning, USA) as described previously [11]. In brief, hGH–P5 K, TT, and OVA were used to measure the anti-PEG, anti-TT, and anti-OVA antibodies, respectively. The three samples were at a protein concentration of 3 μg/ml (100 μl in 50 mM $NaHCO_3$, pH 9.6) and used for well coating at 4 °C overnight. The wells were blocked by 100 μl of PBS buffer (pH 7.4) containing 4% skimmed milk solids by weight (PBS–milk) and washed with PBS buffer (pH 7.4). The serum samples were diluted with PBS–milk in a series of 2-fold dilutions starting from 1:100. The diluted serum samples (100 μl) were added and incubated at 37 °C for 2 h. The wells were emptied and washed with PBS buffer (pH 7.4) containing 0.1% Tween 20 (PBST). Tagging was carried out by HRP-anti-IgM, HRP-anti-IgG, HRP-anti-IgG1, HRP-anti-IgG2a, HRP-anti-IgG2b, and HRP-anti-IgG3, respectively. After washing wells with PBST, 100 μl of chromogenic reagent containing 0.015% (w/v) of TMB was added to the wells and incubated at 37 °C for 30 min. The peroxidase reaction was arrested with 25 μl of 2 M H_2SO_4. The resultant solution was determined spectrometrically at 450 nm. Antibody titers were defined as the dilution number yielding an OD value of 0.2.

2.16. Statistical analysis

One-way ANOVA was utilized to compare the differences between the experimental groups. Results were analyzed using GraphPadPrism 5 software (GraphPad Software, San Diego, CA, USA). The values of $P < 0.05$ (*) and $P < 0.01$ (**) were considered statistically and highly statistically significant between the experimental groups, respectively.

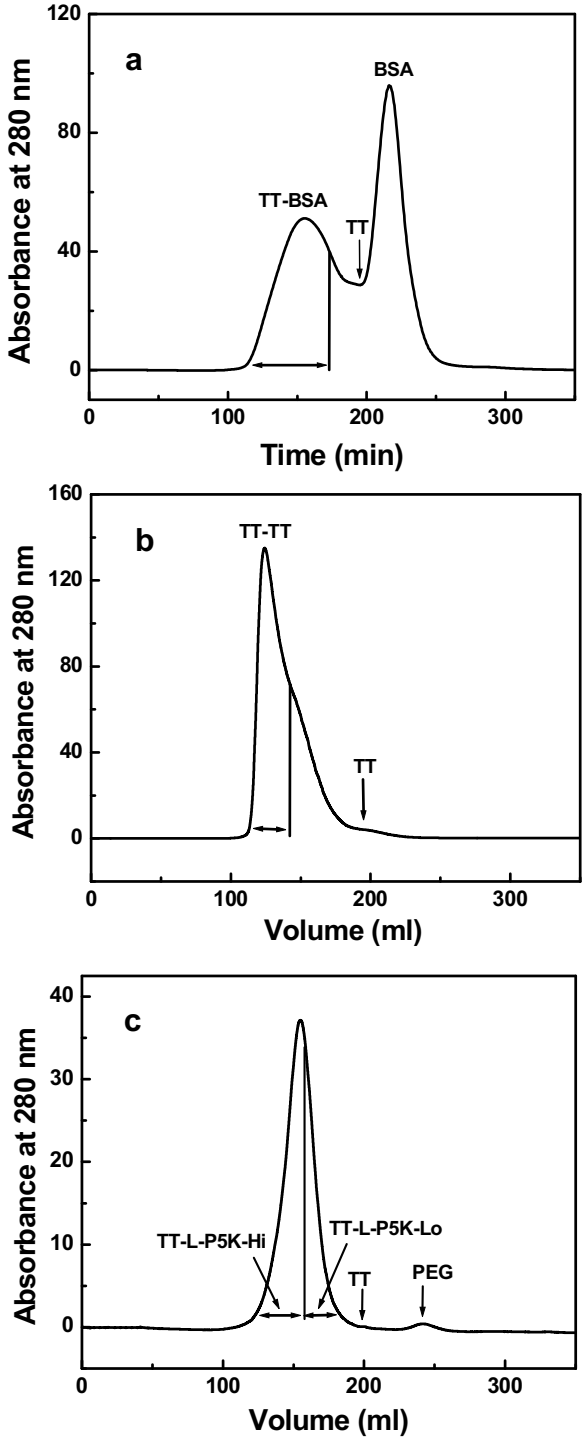

Fig. 1. Purification of TT–BSA, TT–TT, and TT–L–P5 K. TT–BSA (a), TT–TT (b), and TT–L–P5 K (c) were purified from the reaction mixtures by a Superdex 200 column (2.6 cm × 60 cm) at room temperature. The column was equilibrated and eluted by 20-mM phosphate buffer (pH 7.2) at a flow rate of 2.0 ml/min.

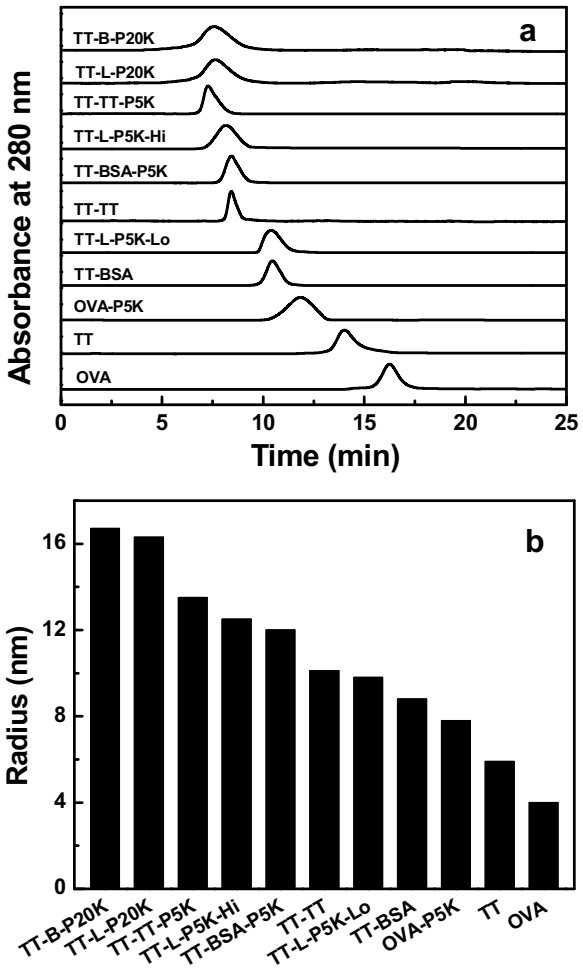

Fig. 2. Characterization of the PEGylated proteins. The PEGylated proteins were analyzed by an analytical Superdex 200 column (1.0 cm × 30 cm) at room temperature (a). The molecular radii were measured by dynamic light scattering (b).

3. Results

3.1. Purification of TT–BSA, TT–TT, and the PEGylated proteins

TT–BSA, TT–TT, and TT–L–P5K were fractionated from the reaction mixtures, using a Superdex 200 column (2.6 cm × 60 cm). As shown in Fig. 1a, two partially resolved elution peaks were observed. TT–BSA was fractionated as indicated by the arrow. As shown in Fig. 1b, a major asymmetric elution peak was observed for TT–TT. TT–TT was fractionated as indicated by the arrows. As shown in Fig. 1c, the PEGylated TT (TT–L–P5K) was eluted as a single and broad peak on the column. The broad elution peak reflected a wide Mw distribution of TT–L–P5K. It also indicated that TT–L–P5K was composed of TT–PEG conjugates containing different PEG amounts. Two components of the elution peak (TT–L–P5K–Hi and TT–L–P5K–Lo) were fractionated as indicated by the arrow.

The PEGylated OVA (OVA–P5K), the PEGylated TT (TT–L–P20K and TT–B–P20K), the PEGylated TT–BSA (TT–BSA–P5K), and the PEGylated TT–TT (TT–TT–P5K) were purified from the reaction mixtures by a tangential flow filtration, using a 50–kDa or a 100–kDa cutoff membrane to remove the free PEG.

3.2. 2 SEC analysis

The purified PEGylated proteins were analyzed on an analytical Superdex 200 column (1 cm × 30 cm). TT was eluted as a single and symmetric peak at 14.0 min (Fig. 2a). TT–TT and TT–BSA were eluted as single peaks at 8.5 min and 10.5 min, respectively. This suggested that conjugation of BSA could increase the hydrodynamic volume of TT. In contrast, the elution peak of TT–TT–P5 K and TT–BSA–P5K was at 7.2 min and 8.4 min, respectively. OVA was eluted as a single peak at 16.3 min. The elution peak of OVA–P5K (11.9 min) was left-shifted as compared to that of OVA. The elution peak of TT–L–P5K–Hi (8.7 min) was left-shifted as compared

to that of TT–L–P5K–Lo (10.4 min). Moreover, the elution peaks of TT–L–P20K (7.6 min) and TT–B–P20K (7.6 min) were left-shifted as compared to that of TT–L–P5K–Hi (8.7 min).

3.3. Dynamic light scattering

The molecular radii of the PEGylated proteins were measured by DLS. Size distributions of the samples were found to be unimodal, and the centered point was used to obtain the molecular radius. The peak was in the range of 98.5–100% intensity and 99.0–100% volume.

As shown in Fig. 2b, the molecular radius of TT–BSA (8.8 nm) was higher than that of TT (5.9 nm) and lower than that of TT–BSA–P5K (12.0 nm). The molecular radius of TT–TT (10.1 nm) was higher than that of TT (5.9 nm) and lower than that of TT–TT–P5K (13.5 nm). The molecular radii of TT–L–P5K–Hi (12.5 nm), and TT–L–P5K–Lo (9.8 nm) were higher than that of TT. The molecular radius of OVA-P5K (7.8 nm) was higher than that of OVA (4.0 nm). This suggested that PEGylation significantly increased the molecular radii of the proteins. Moreover, the molecular radii of TT–L–P20K (16.4 nm) and TT–B–P20K (16.7 nm) were higher than that of TT–L–P5K–Hi (12.5 nm).

3.4. Quantitative analysis

The PEG amounts of the PEGylated proteins were quantitatively analyzed. The molar ratios of PEG to TT in TT–L–P5K–Hi, TT–L–P5K–Lo, TT–L–P20K, and TT–B–P20K were 24.0 ± 2.7, 16.9 ± 2.1, 14.8 ± 1.6, and 14.0 ± 0.9, respectively. Thus, the PEGylation extent of TT–L–P5K–Lo was lower than that of TT–L–P5K–Hi and comparable to those of TT–L–P20K and TT–B–P20K. In contrast, the molar ratios of PEG to protein in TT–BSA–P5K, TT–TT–P5K, OVA–P5K, and hGH–P5K were 15.6 ± 2.3, 14.8 ± 2.3, 6.6 ± 0.8, and 6.3 ± 0.6, respectively.

3.5. Circular dichroism spectroscopy

Far-UV CD spectroscopy was carried out to investigate the secondary structures of the samples. As shown in Fig. 3, the CD spectra of TT and OVA both exhibited doublet bands at 208 nm and 222 nm, respectively. This indicated rich α-helix and β-sheet contents of TT and OVA. The CD spectra of TT–L–P5K–Hi, TT–L–P5K–Lo, TT–TT–P5 K, and TT–TT were almost superimposed on that of TT. The CD spectra of OVA–P5 K and TT–BSA–P5 K were similar to those of OVA and TT–BSA, respectively. This indicated that the secondary structures of the proteins (OVA, TT, and TT–BSA) were not altered upon PEGylation of these proteins.

3.6. Anti-protein antibodies

The anti-TT antibody titers in the mouse sera were measured by ELISA. As shown in Fig. 4a, the anti-TT IgG titers in the TT group were lower than those in the TT–TT group ($P < 0.05$). This suggested that TT–TT induced stronger anti-TT immune response than TT. The anti-TT IgG titers in the TT group were higher than those in the TT–BSA group ($P < 0.05$). This indicated that the anti-TT immune response to TT was decreased by conjugation of BSA. Consistently, conjugation of albumin can reduce the immunogenicity of a protein (e.g., urokinase) by masking the antigenic epitopes on the protein surface [24]. The anti-TT IgG titers in the TT–TT–P5K group were higher than those in the TT–L–P5K–Hi and TT–L–P5K–Lo groups ($P < 0.05$). The anti-TT IgG titers in the TT–L–P5K–Lo group were higher than those in the TT–L–P5K–Hi group ($P < 0.05$). In addition, the anti-OVA IgG titers in the OVA–P5K group were lower than those in the OVA group ($P < 0.05$, inset, Fig. 4a).

3.7. Anti-PEG antibodies

3.7.1. Repeated dose effect on the anti-PEG antibodies

The anti-PEG antibody titers in the mouse sera were measured by ELISA. The PEGylated proteins elicited low anti-PEG IgG titers at the first dose (on day 7, Fig. 4b). The third dose (on day 21, Fig. 4b) and the fifth dose (on day 35, Fig. 4c) significantly increased the anti-PEG IgG titers elicited by the PEGylated proteins. Compared to those at the first dose, the anti-PEG IgG titers elicited by TT–BSA–P5 K, TT–TT–P5K, TT–L–P5K–Hi, TT–L–P5K–Lo, and OVA–P5K at the third dose showed 3.5-, 8.0-, 8.0-, 5.7-, and 4.0-fold increase, respectively. Moreover, the anti-PEG IgG titers elicited by TT–BSA–P5K, TT–TT–P5K, TT–L–P5K–Hi, TT–L–P5K–Lo, and OVA–P5K at the fifth dose showed 6.9-, 16.0-, 32.0-, 90.8-, and 8.0-fold increase, respectively. This reflected a classical immunological response toward a reintroduced antigen. Moreover, TT–L–P5K–Lo elicited the highest anti-PEG IgG titers among the five PEGylated proteins.

3.7.2. Effect of proteins on the anti-PEG antibodies

Although the extent of PEGylation in TT–L–P5K–Lo was comparable to those in TT–BSA–P5K and TT–TT–P5K, the anti-PEG IgG titers in the TT–L–P5K–Lo group were higher than those in the TT–BSA–P5K and the TT–TT–P5K groups ($P < 0.05$, Fig. 4c). In contrast, the anti-TT immune response to TT–L–P5K–Lo was lower than that to TT–TT–P5K and higher than that to TT–BSA–P5K. Thus, the anti-PEG immune response to a PEGylated protein may be affected by the residual immunogenicity of the protein in the PEGylated protein. Moreover, this indicated that the immunogenicity of the protein in the PEGylated protein might be the determining factor.

3.7.3. Effect of the extent of PEGylation on the anti-PEG antibodies

Although the extent of PEGylation in TT–L–P5K–Lo was lower than that in TT–L–P5K–Hi, the anti-PEG IgG titers elicited by TT–L–P5K–Hi and TT–L–P5K–Lo were comparable to each other on days 7 and 21 (Fig. 4b). However, the anti-PEG IgG titers in the TT–L–P5K–Lo group were much higher than those in the TT–L–P5K–Hi group on day 35 ($P < 0.05$, Fig. 4c), even though TT–L–P5K–Lo showed a lower extent of PEGylation than TT–L–P5K–Hi.

3.7.4. Anti-PEG IgG subclass

Anti-PEG IgG subclass titers were measured by ELISA. All the PEGylated proteins elicited major IgG1 titers that were close to their corresponding IgG titers (Fig. 4d). The anti-PEG IgG1 titers in the TT–L–P5K–Lo group were the highest among the five groups. In contrast, the anti-PEG IgG2a, IgG2b, and IgG3 were almost undetectable. More importantly, high titers of anti-PEG IgG1 were observed for all the PEGylated proteins.

3.7.5. Effect of the Mw and branching of mPEG on the anti-PEG antibodies

As shown in Fig. 5a, the anti-TT IgG titers in the TT–L–P5K–Lo group were lower than those in the TT group ($P < 0.05$) and higher than those in the TT–L–P20K and TT–B–P20K groups on days 14 and 35 ($P < 0.05$). However, the anti-TT IgG titers in the TT–L–P20K group were comparable to those in the TT–B–P20K group.

As shown in Fig. 5b, the anti-PEG IgG titers elicited by TT–L–P20K were higher than those by TT–L–P5K–Lo ($P < 0.05$). Because the number of the conjugated mPEG was comparable between TT–L–P20K and TT–L–P5K–Lo, this indicated that the Mw of mPEG showed a strong effect on the anti-PEG immune response. In contrast, the anti-PEG IgG titers elicited by TT–L–P20K were compared with those by TT–B–P20K. This suggested that the

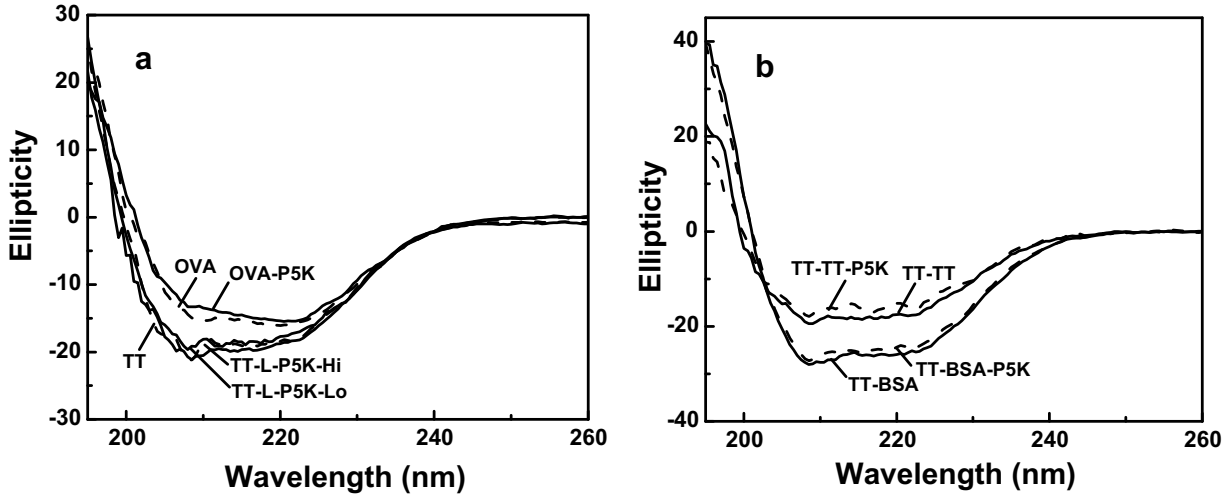

Fig. 3. Circular dichroism spectra of the PEGylated proteins. CD spectra were recorded between 260 nm and 190 nm at room temperature.

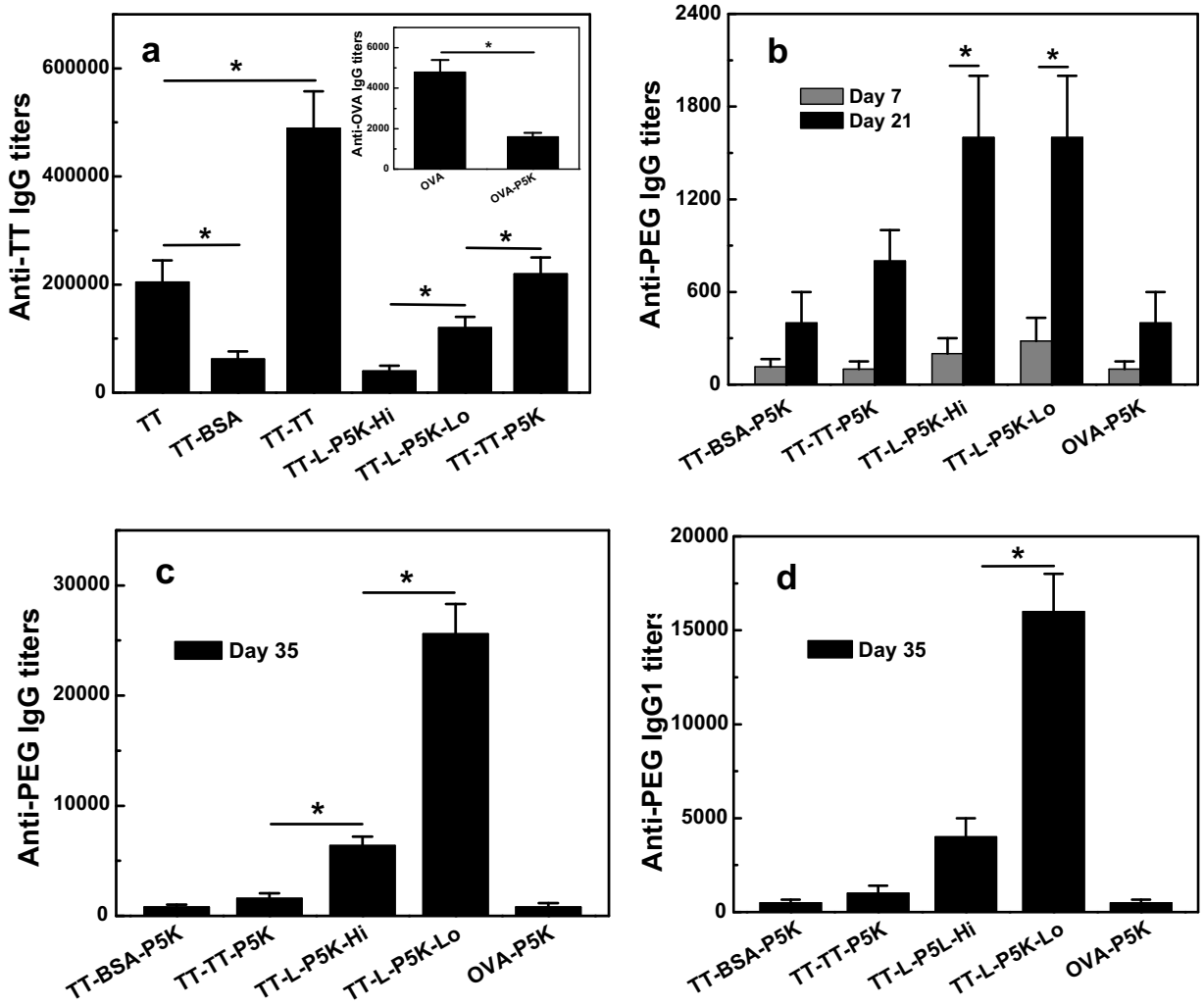

Fig. 4. Antibody response after immunization of the PEGylated proteins. Anti-TT IgG on day 35 (a), anti-OVA IgG (inset, a), anti-PEG IgG on days 7 and 21 (b), anti-PEG IgG on day 35 (c), and anti-PEG IgG1 on day 35 (d) were measured using ELISA. Values represent mean ± S.D. from 6 mice per group.

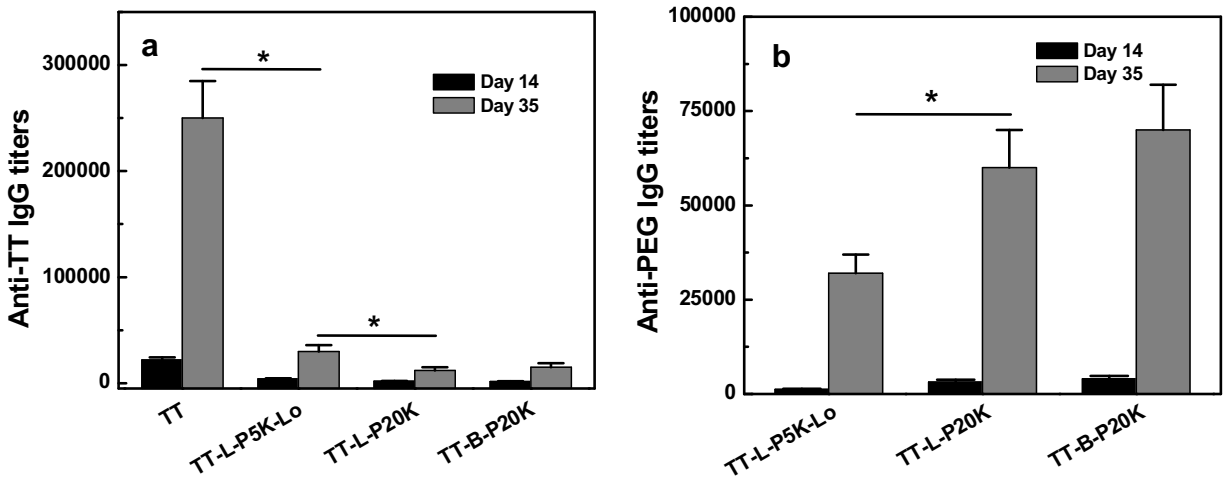

Fig. 5. Antibody response after immunization of the PEGylated proteins. Anti-TT IgG (a) and anti-PEG IgG (b) on days 14 and 35 were measured using ELISA. Values represent mean ± S.D. from 6 mice per group.

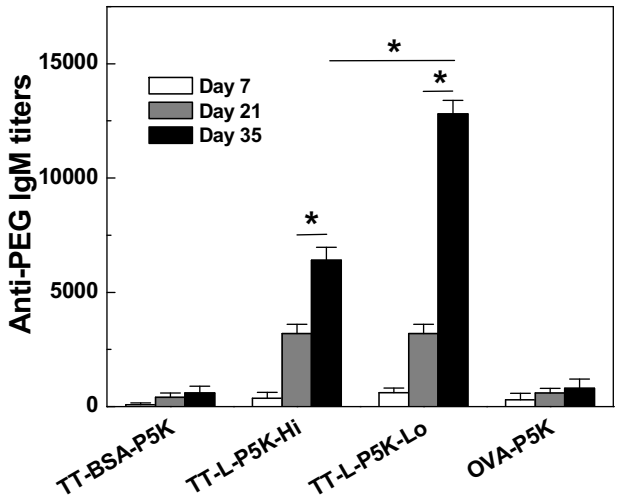

Fig. 6. Antibody response after immunization of the PEGylated proteins. Anti-PEG IgM were measured using ELISA. Values represent mean ± S.D. from 6 mice per group.

branching of mPEG had an insignificant effect on the anti-PEG immune response.

3.7.6. Anti-PEG IgM

As shown in Fig. 6, the PEGylated proteins elicited low anti-PEG IgM titers on day 7. The anti-PEG IgM titers in all the groups increased on day 21. In contrast, the anti-PEG IgM titers in the TT–BSA–P5K and OVA–P5K groups were essentially not altered on day 35. However, the anti-PEG IgM titers in the TT–L–P5K–Hi and TT–L–P5K–Lo groups significantly increased on day 35. Moreover, the anti-PEG IgM titers in the TT–L–P5K–Lo group were higher than those in the TT–L–P5K–Hi group ($P < 0.05$).

4. Discussion

The main objective of the present study was to investigate the effect of protein immunogenicity, PEG size and PEG branching on the anti-PEG immune response to the PEGylated proteins. Here, the PEGylated proteins (TT, TT–BSA, and OVA) were used to investigate their anti-PEG immunological properties.

The amines of TT were converted to thiols for preparation of TT–BSA. The thiolated TT was conjugated with maleimide groups of the EMCS–modified BSA. Subsequently, the maleimide mPEG reacted with the thiols of TT to generate TT–BSA–P5K, where mPEG was specifically conjugated with TT portion of TT–BSA. BSA was not PEGylated and far from mPEG, which significantly decreased the steric shielding effect of BSA on PEG. Thus, the lower anti-PEG immune response to TT–BSA–P5K was not due to the steric shielding effect of BSA on mPEG. In contrast, the NHS chemistry led to PEGylation of TT and BSA, which rendered a strong steric shielding effect of BSA on mPEG. Thus, maleimide chemistry-based PEGylation was used to prepare the PEGylated proteins.

The TT–PEG conjugate showed a very wide symmetrical peak in the SEC chromatogram (Fig. 1c), which reflected the heterogeneity of the conjugate. The former part in the elution peak (TT–L–P5K–Hi) represented a population of TT–L–P5K with a higher extent of PEGylation. The latter part in the elution peak (TT–L–P5K–Lo) represented a population of TT–L–P5K with a lower extent of PEGylation.

The succinimidyl thioether was the chemical bond in TT–BSA and the PEGylated proteins, which was obtained by Michael-addition of a thiol to a maleimide. A recent study suggested that the ultimate in vivo fate of such conjugates was to undergo disruptive cleavage by thiol exchange or stabilizing ring opening [25]. If the succinimide moiety of a maleimide–thiol conjugate was hydrolyzed, the ring-opened product was stabilized toward the cleavage with half-lives of over 2 years [25]. Here, a large amount of PEG-bound water molecules can facilitate the hydrolysis of the chemical bond, thereby increasing the in vivo stability of the conjugate. In addition, the thiol exchange occurs mainly in cells, because of the intracellular presence of thiol-containing molecules such as glutathione. Cleavage of the PEGylated proteins may be significantly decreased in the plasma. Thus, the bond stability has an insignificant effect on the immunogenicity of the PEGylated proteins.

Because hGH is a heterologous protein to the BALB/c mice and has not been used to immunize the mice, the non-specific binding of the species in the serum to hGH should be very low. In addition, hGH-P5K was prepared using succinimide chemistry, which was different from the maleimide chemistry used in the present study. This will decrease the non-specific binding to hGH–P5K resulting from the chemical bond.

It should be mentioned that Tween 20 containing more than 70% PEG was used to wash the wells of ELISA plates in the present study. This possibly interfered with the binding of anti-PEG antibodies to the coating on the ELISA wells. However, anti-PEG antibodies in

the serum samples were tightly bound to hGH–P5K in the coating before washing of the ELISA wells with 0.1% Tween 20. Moreover, the methoxy moiety was present in hGH–P5K and absent in Tween 20; hGH–P5K showed much higher binding affinity to the anti-PEG antibodies than the PEG in Tween 20. Thus, these two factors could minimize the effect of Tween 20 on the presented results.

Our present study suggested that the PEGylated proteins mainly elicited the anti-PEG antibodies of IgM and IgG1. The anti-PEG IgM elicited by the PEGylated proteins is in a manner similar to that of the PEGylated liposome [26,27]. The PEG moiety in the PEGylated liposome crosslinks the cell–surface immunoglobulins of specific B cells and triggers B cell receptor-mediated endocytosis of the PEGylated liposome, thereby producing massive amounts of anti-PEG IgM [27]. The anti-PEG IgG1 elicited by the PEGylated proteins is in a manner similar to anti-polysaccharide (PS) IgG1 elicited by the PS–protein conjugate vaccine [28]. The immunogenic carrier protein is capable of imparting immunogenicity to PS. Proteolysis and presentation of the protein epitopes confer the anti-PS B cells the ability to activate T helper cells and facilitate the substantial secretion of anti-PS IgG1 by the B lymphocytes [29].

Previously, anti-PEG antibodies in rabbits were raised by immunization with PEGylated proteins in FCA [12]. In contrast, the PEGylated TT samples were used for immunization in the absence of adjuvants and elicited a strong anti-PEG immune response. This avoided the interference of adjuvants in the anti-PEG immune response. Moreover, the effects of Mw and branching of mPEG have been investigated in our study. Recently, a protein modification technology has been developed by individual encapsulation of a therapeutic protein in a superhydrophilic zwitterionic gel [30]. Interestingly, the immune response to either the protein or the polymer was not observed following repeated injections. The authors hypothesized that an extremely hydrophilic polymer should do a better job than the amphiphilic PEG for modification of proteins [30].

Usually, the PEGylated therapeutic proteins (e.g., IFN–α–PEG40K and G–CSF–PEG20K) contain one mPEG chain, which helps retain the proteins' activity. However, these PEGylated proteins have not been observed to induce anti-PEG immune response. For the PEG–uricase (Krystexxa), uricase was conjugated with 36–40 PEG10K chains. In the present study, TT was conjugated with ~26 PEG5K chains. Thus, the present conjugate was close to the clinically relevant mPEG levels in PEG–uricase. TT and OVA were typically used for the PS–protein conjugate vaccines [28,31] and acted as the target proteins in the present study. Although these PEGylated proteins have no significant advantages in clinical applications, they are good candidates to investigate the anti-PEG immune response to the PEGylated proteins.

5. Conclusion

In summary, our result suggested that the anti-PEG immune response to the PEGylated proteins depended on the immunogenicity of proteins, the extent of PEGylation, and the Mw of mPEG. In contrast, the branching of mPEG had an insignificant effect on the anti-PEG immune response to the PEGylated proteins.

Acknowledgments

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