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Purification of antigenized immunoglobulins derivatized with monomethoxypolyethylene glycol

T.-D. Brumeanu, H. Zaghouani[1], C. Bona*

Department of Microbiology, Mount Sinai School of Medicine, Box 1124, New York, NY 10029, USA

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Abstract

Genetically engineered immunoglobulins (Igs) carrying viral B or T cell peptides in the CDR3 loop, function as efficient delivery system of the defined viral epitopes. Two of these antigenized Igs (AIgs) were derivatized with 2-O-monomethoxypolyethylene glycol-4,6-dichloro-*s*-triazine (mPEG). Herein, we describe a two-step strategy to purify mPEG-derivatized AIgs (AIgs–mPEG). Unreacted mPEG polymers were removed by size-exclusion chromatography using ammonium hydrogencarbonate as a buffer system. Mildly PEGylated AIgs were isolated from free and highly derivatized AIgs by anion-exchange chromatography. Electrophoretic analysis indicated that the AIgs–mPEG preparation contained less than $4 \cdot 10^{-4}$ *M* unreacted mPEG. This strategy may be applied to other mPEG-derivatized monoclonal antibodies.

1. Introduction

Polyethylene glycol (PEG) is non-toxic, non-immunogenic, and is approved by the US Food and Drug Administration (FDA) for internal use in humans. PEG is commonly used as additive to food and drugs. PEGylated proteins preserve their biologic activity to various extends depending on the degree of PEGylation. Bovine serum catalase derivatized with 2-O-monomethoxypolyethylene glycol-4,6-dichloro-*s*-triazine (mPEG) at low ratio retained full enzymatic activity and it was more stable and soluble in aqueous solutions [1,2]. Derivatization of 7–14% of the primary amines of proteins, significantly increased the half life in blood circulation without loss of biologic activity [3]. Highly mPEG-derivatized immunoglobulins (18–25%) exhibited tolerogenic effect in mice [4]. Administration of PEGylated self peptides to mice with experimental autoimmune myasthenia Gravis suppressed the production of acetylcholine receptor specific autoantibodies [5].

The increase in half life of proteins modified with mPEG was correlated with high resistance to enzymatic degradation and low clearance rate through the kidneys [6]. Based on these observations many of the mPEG-derivatized enzymes, cytokines and monoclonal antibodies entered clinical trials as substitutes or immunotherapeutic agents [7–11].

Genetically engineered Ig molecules carrying viral B or T cell epitopes in the CDR3 loop can

* Corresponding author.

[1] Present address: University of Tennessee, Department of Microbiology, M409 Walters Life Science, Knoxville, TN 37996, USA.

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prime animals for the induction of specific immune responses [12–16]. Ig-HA is a BALB/c IgG2b antibody expressing in the CDR3 loop a T cell epitope (HA110–120) from the hemagglutinin of PR8 influenza virus [13]. Ig-HA was able to prime BALB/c mice and to mount virus-specific T helper response [13]. Ig-V_3C is a mouse variable-human γ_1/k constant regions, expressing within the heavy-chain CDR3 loop a B cell epitope from the V_3 cysteine bridged loop of the envelope protein of HIV-1. Ig-V_3C was able to induce virus-specific antibody response in baboons [16]. Herein we derivatized Ig-HA and Ig-V_3C with mPEG 5000 and attempted to purify the antigenized Igs (AIgs)–mPEG conjugates.

During the PEGylation process, various degrees of derivatization may occur as a consequence of the micro heterogeneity of the protein, the distribution of both the number and the position of attachment of PEG units, the inherent polydispersity of PEG polymers, and microenvironmental conditions of the reaction. In addition, dioxy-PEG that can cross-link proteins maybe present in commercial preparation as a result of hydrolysis of the ethylene oxide monomers during polymerization process [17,18]. Thus, protein–mPEG preparations may contain species of highly PEGylated and/or cross-linked proteins in addition to unreacted materials. Purification of the conjugates is therefore required prior to their use in biological systems. Purification of protein–PEG conjugates has always relayed on a single-step chromatographic removal of free PEG polymers [3,6,19,20]. Herein, we describe a two step-procedure able to remove free mPEG, free AIgs and heavily PEGylated AIgs leading to a relatively pure population of mildly PEGylated AIgs.

2. Experimental

2.1. Chemicals

AIgs (Ig-HA and Ig-V_3C) were generated in our laboratory as previously described [12–16]. PhastSystem electrophoresis apparatus, PhastGels 4–15% gradient of polyacrylamide and cyanogen bromide-activated Sepharose CL-4B were purchased from Pharmacia-LKB. Ultrogel AcA-44 and dialysis bags of 75 000 MWCO (molecular weight cut-off) were from Spectrum. Ultra concentrators of 100 000 MWCO (molecular weight cut-off) (Centrex UF-2, 2 ml volume) were from Schleicher & Schuell. Rat hybridoma cells secreting monoclonal anti-murine k chain antibodies and HP6053 mouse hybridoma producing anti-human k chain antibodies were from ATCC. The Q300 HPLC column (250 × 4.6 mm, 300 μm particle size) was from Rainin. Nessler's reagent and 2,4,6-trichloro-*s*-triazine-activated monomethoxypolyethylene glycol MWCO 5000 (mPEG 5000) were from Sigma. The agarose gels (Titan gel high-resolution protein kit) were from Helena Labs.

3.2. Purification of AIgs

Ig-HA and Ig-V_3C were affinity purified on a rat anti-murine k chain and mouse anti-human k chain antibody-Sepharose columns, respectively. Affinity purified AIgs were equilibrated in 0.1 *M* sodium tetraborate, pH 9.6 and concentrated to 1 mg/ml by ultracentrifugation using tubes of 100 000 MWCO.

3.3. Derivatization of AIgs with mPEG

A 10-mg amount of each AIg was derivatized with 2,4,6-trichloro-*s*-triazine-activated mPEG 5000 as described [20]. Briefly, a 50 times molar excess of mPEG was added to 10 mg AIgs in 10 ml of 0.1 *M* tetraborate buffer, pH 9.6. The mixture was stirred vigorously for 4 h at room temperature. The conjugate preparations were concentrated to 1.5 ml in tubes of 100 000 MWCO and further purified.

3.4. Chromatographic purification of AIg–mPEG conjugates

AIg–mPEG preparations were applied on an Ultrogel AcA-44 gel filtration column (80 × 1.6 cm) equilibrated with 0.1 *M* NH_4HCO_3, pH 8.5 and flow-rate of 0.4 ml/min. Fractions were collected at 4-min intervals, dried by speed vacuum centrifugation and resuspended in 0.5 ml

of 5 m*M* sodium acetate, pH 5. Each fraction was then analyzed for protein content by Biuret micro assay, and for the presence of free hydrolyzed mPEG by Nessler's reagent as described [4]. Fractions of the conjugates free of hydrolyzed mPEG were then rechromatographed on a Q300 anion-exchange HPLC column equilibrated with 5 m*M* sodium acetate, pH 5 using a 45-min linear gradient from 5 to 500 m*M* sodium acetate, pH 5, and flow-rate of 0.5 ml/min. Fractions from Q300 column were dialyzed in Spectrapor bags with 75 000 MWCO against phosphate-buffered saline (PBS) and concentrated in Centrex UF-2 tubes.

3.5. Electrophoretic analyses of AIg–mPEG conjugates

The purity of AIg–mPEG conjugate was analyzed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) and mPEG was detected by electrophoresis on agarose gels. The SDS-PAGE was performed under non-reducing conditions as described by Laemmli [21]. Briefly, samples (5 μg) were resuspended in 5 μl of sample buffer (0.1% SDS, 0.1 *M* Tris–HCl, 6 *M* urea, pH 8.5) and incubated at 37°C for 2 h. Samples were run on 4–15% gradient PhastGel (Pharmacia) at 250 V and 5 W for 45 min at 15°C. The gel was then fixed and silver stained. Electrophoresis on agarose Titan gels was carried out according to the manufacture's instructions. Samples (10 μg in 5 μl barbital buffer) were run at 250 V for 30 min on duplicate gels. One gel was stained with a mixture of 0.1% Coomassie Blue R-250 and 1% Ponceau S, and the second gel was immersed in 20% trichloroacetic acid (TCA) in water for 30 s and immediately photo-scanned.

3.6. Fluorescamine assay

Graded amounts (1, 0.5 and 0.25 μg) of AIg–mPEG or AIgs in 25 μl of PBS were mixed with equal volume of fresh fluorescamine in acetone (150 mg/ml) and incubated for 5 min at room temperature. Samples were then brought to 1 ml with PBS and fluorescent intensity was measured at 475 nm emission versus 390 nm excitation. The percent of PEGylated primary amines was estimated according to the following formula $1 - (A_{475}$ AIgs–mPEG$/A_{475}$ AIgs$) \times 100$ [22].

4. Results and conclusions

4.1. Derivatization of AIgs with mPEG

Conjugation of proteins with mPEG increase the half life, solubility, and stability of the proteins. The biological activity may be reduced depending on the degree of PEGylation [1,2,6,7]. Igs engineered to carry viral peptides in the CDR3 loop are efficient delivery systems of viral epitopes to the immune system and induce virus-specific responses [12–16].

Preliminary experiments indicated that derivatization of 6–8% of the primary amines of AIgs with mPEG requires 50-fold molar excess of mPEG. Following the derivatization procedure described in the Experimental section, we PEGylated 10 mg of both Ig-HA and Ig-V_3C with mPEG. The chemical events involved in this PEGylation process are described in Fig. 1. mPEG has an active chlorine that can be substituted with the ϵ amino group of lysine residues at 18°C and pH 9.6. Since hydrolysis is faster than aminolysis, an excess of mPEG is required to achieve optimal coupling. A significant amount of hydrolyzed mPEG is thus generated. The reactivity of commercial mPEG 5000 varies from batch to batch, leading to different degrees of PEGylation. Preliminary experiments of PEGylation of bovine γ-globulins with the batch used in these studies indicated that the molar ratio Ig/mPEG of 1 to 50 was optimal to achieve 6 to 8% substitution of the primary amines.

Each of the AIg–mPEG preparation contained excess of free hydrolyzed PEG, unconjugated AIg, mildly and heavily conjugated AIgs. Only mildly PEGylated AIgs were of interest for our immunological studies. Thus, all other compounds had to be removed in order to perform pertaining evaluation of the effect of mild PEGylation on the immunogenicity of viral peptides grafted in the Igs.

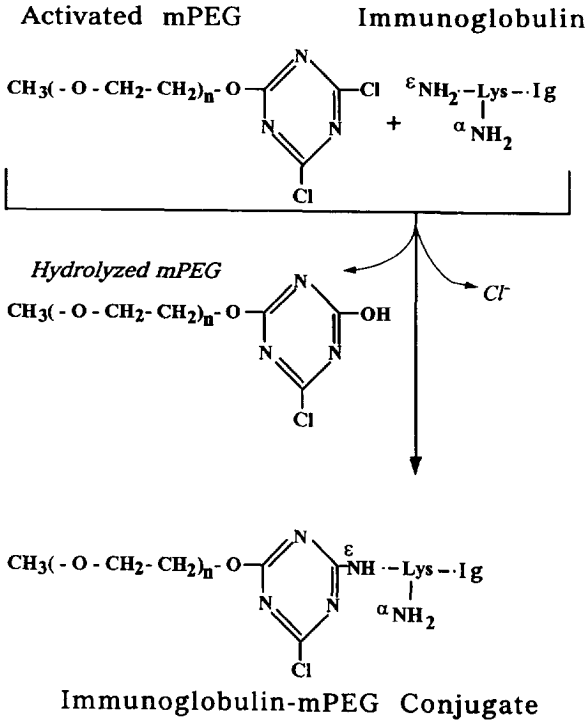

Fig. 1. Chemical reactions for the attachment of mPEG to AIgs.

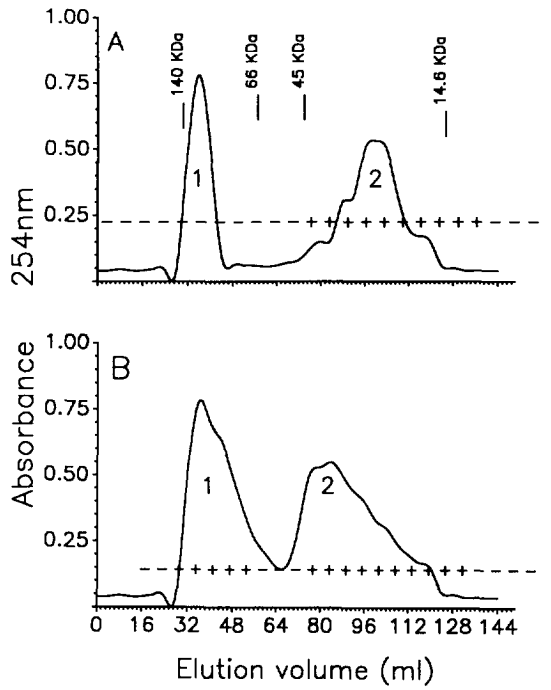

Fig. 2. Removal of hydrolyzed mPEG from AIg-mPEG preparation by size-exclusion chromatography. Ultrogel AcA-44 column (80 × 1.6 cm) was calibrated at 0.4 ml/min flow-rate with molecular mass markers (Pharmacia-LKB) and then loaded with 10 mg of Ig-HA–mPEG preparation. The column was equilibrated with either 0.1 *M* NH_4HCO_3 (A) or PBS (B). Peak 1 contained the conjugates and peak 2 corresponds to free mPEG. A plus sign indicates the presence of free polymer as detected by Nessler's test. KDa = kilodalton.

4.2. Chromatographic removal of free mPEG from AIg–mPEG preparations

During the conjugation process significant amounts of hydrolyzed mPEG are generated. Removal of excess of free mPEG is the foremost step for purification of AIgs–mPEG conjugates. To this aim, samples were applied on Ultrogel AcA-44 equilibrated in ammonium hydrogencarbonate. AIg–mPEG conjugates were eluted in the exclusion volume and were tested for the presence of free PEG using Nessler's reagent. As indicated in Fig. 2, AIgs–mPEG as well as unconjugated AIgs are eluted in the exclusion volume (peak 1). Free hydrolyzed mPEG eluted in later fractions (peak 2). It should be noted that free mPEG which is of M_r 5000 has an unusual elution profile between M_r 15 000–45 000 in agreement with previous observations [17,23]. Equilibration of the column with ammonium hydrogencarbonate allowed for a better removal of free mPEG than the equilibration with PBS, as indicated by Nessler's test. The improved resolution obtained with ammonium hydrogencarbonate maybe related to better neutralization of the active charges on gel matrix and therefore lower interactions between free mPEG polymer and the matrix.

4.3. Removal of the unreacted compounds from AIg–mPEG conjugates

Because no one-step procedure for the separation of protein–mPEG conjugates from unreacted compounds is described, we chose a method that includes a first-step removal of hydrolyzed mPEG and a subsequent step that

removes underivatized and highly derivatized AIgs. Since the size-exclusion limit of the Ultrogel AcA-44 column is 140 000 kDa, hydrolyzed mPEG can be removed but the underivatized AIgs can not. The removal of excess mPEG is required because it could interfere with the isolation of the conjugates in the subsequent anion-exchange chromatography step. Preliminary experiments performed on anion-exchange HPLC columns as a single step of purification, showed poor resolution and low yields even if minimal amounts of sample were applied on the column. In fact, the conjugates showed a broad elution profile as a result of the presence of excess of free polymers which interfere strongly with the binding of conjugates to the anion-exchange matrix. Poor resolution of PEGylated proteins was also described when conjugates were separated by charge-reversal capillary zone electrophoresis without previous removal of free PEG [19,23]. The authors concluded that it is of great importance to remove free polymers from the conjugate preparation in order to obtain good resolution in different separating media.

To isolate mildly PEGylated AIgs with 6–8% degree of PEGylation, we rechromatographed the conjugates on anion-exchange HPLC column under optimized conditions as described in the Experimental section. Fig. 3 shows the elution profiles of Ig-HA–mPEG (Fig. 3a) and free Ig-HA (Fig. 3b). In the case of Ig-HA–mPEG, three major peaks were eluted from the column and labeled 1, 2 and 3 (Fig. 3a). Peak 3 represents free Ig-HA since it elutes at the same salt concentration as the unconjugated control Ig-HA. The material of peak 1 may represents highly PEGylated AIgs that could not bind to the matrix. Peak 2 seems to contain mildly PEGylated Ig-HA as revealed by SDS-PAGE analysis.

4.4. Analysis of the purity of AIg–mPEG conjugates

The purity of AIgs–mPEG preparations was further analyzed for the presence of traces of free mPEG and unconjugated AIgs by electrophoresis on agarose and polyacrylamide gels,

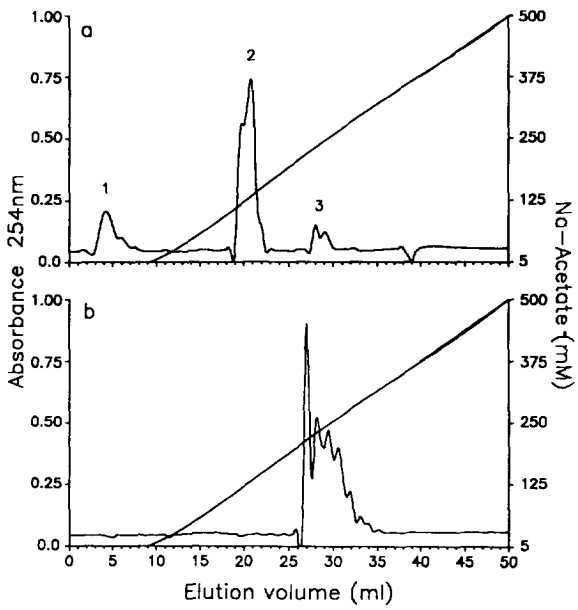

Fig. 3. Removal of un-PEGylated AIgs from AIg–mPEG preparations by anion-exchange HPLC. The material of peak 1 in Fig. 2, presumably Ig-HA–mPEG and other materials (a) and un-PEGylated Ig-HA preparation (b) were loaded onto Q300 anion-exchange HPLC column and chromatographed as described in the Experimental section.

respectively. Although purification of AIgs–mPEG conjugates followed one-step purification by size exclusion chromatography, small amounts of free mPEG that could not be detected by Nessler's test may be present in the conjugate preparations. To trace small amounts of free PEG that could interfere with the immunogenicity of the AIgs–mPEG we developed an original, sensitive electrophoretic technique able to detect μM of free mPEG. Based on the observation that mPEG migrate on agarose to the cathode and can be visualized by TCA precipitation but not by protein dyes, we attempted to trace free mPEG in our preparation by electrophoresis on Titan gel followed by TCA precipitation. As can be seen in Fig. 4, left panel, Ig-HA–mPEG preparation obtained from the second chromatographic purification (lane 1) like un-PEGylated Ig-HA preparation (lane 2), did not show detectable amount of free hydrolyzed mPEG while the preparation collected from the first chromatographic purification con-

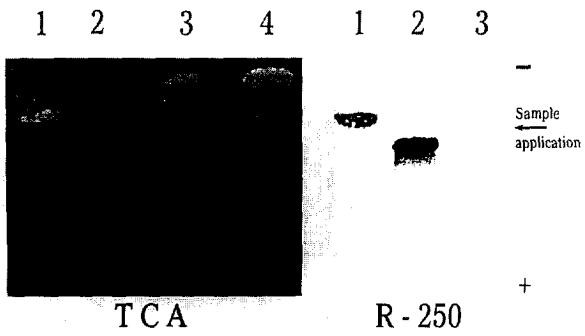

Fig. 4. Detection of residual mPEG in AIgs–mPEG preparations by electrophoresis on Titan agarose gel. Samples collected at various steps of purification were run on duplicate Titan gels and either stained with Coomassie/Ponceau (R250) or precipitated with TCA. Lanes: 1 = Ig-HA–mPEG conjugate after purification on anion-exchange HPLC; 2 = un-PEGylated Ig-HA; 3 = mPEG 5000 ($4 \cdot 10^{-4}$ *M*); 4 = Ig-HA–mPEG conjugate after first step of purification by gel filtration on AcA-44 (not done in Coomassie staining).

tained residual free PEG (lane 4). The amount of free residual mPEG, if any, in the final Ig-HA–mPEG preparation (lane 1) should be lower than $4 \cdot 10^{-4}$ *M*. Calibration experiments indicated that as little as $4 \cdot 10^{-4}$ *M* of mPEG 5000 can be detected using this technique (lane 3).

When a duplicate gel was stained with Coomassie/Ponceau (Fig. 4, right panel), only Ig-HA and Ig-HA–mPEG conjugates were revealed (lanes 1 and 2, respectively) but not free hydrolyzed mPEG (lane 3). Interestingly, PEGylated Ig-HA showed better staining with Ponceau S than un-PEGylated Ig-HA. The differential staining maybe attributed to different ability of the two dyes to access their specific sites on the native protein versus the PEGylated one.

Using this assay, we were also able to trace free mPEG polymers in preparations of chicken egg ovalbumin–mPEG, bovine serum albumin–mPEG and bovine γ-globulin–mPEG conjugates (not shown). Although our electrophoretic technique shows a sensitivity of detection for mPEG similar to that of Childs' assay [24] or the test of Schiavon et al. [25], it has the advantage of distinguishing free PEG from PEG attached to proteins.

To investigate the extend to which AIgs–mPEG preparations were depleted of free and highly PEGylated AIgs, we analyzed the Ig-HA–mPEG and Ig-V_3C–mPEG conjugates by SDS-PAGE on 4–15% gradient gels (PhastGels, Pharmacia) under non-reducing conditions. The results in Fig. 5 show that the final Ig-HA–mPEG and Ig-V_3C–mPEG preparation (peak 2 in Fig. 3) were pure (second and fourth lanes, respectively) and did not contain un-PEGylated Ig-HA and Ig-V_3C. They also did not contain highly PEGylated AIgs. Since the 4–15% gradient gels offer a high resolution for proteins within the range of M_r 50 000–350 000, it is possible to distinguish mild (6–8%) from heavily (15% up) PEGylated AIgs. In Fig. 5, lanes 3 and 6 show the presence of material with $M_r > 250\,000$, indicating that high PEGylation can still take place at a low ratio of 1:50. After anion-exchange HPLC purification, two major populations of Ig-HA–mPEG conjugates migrating

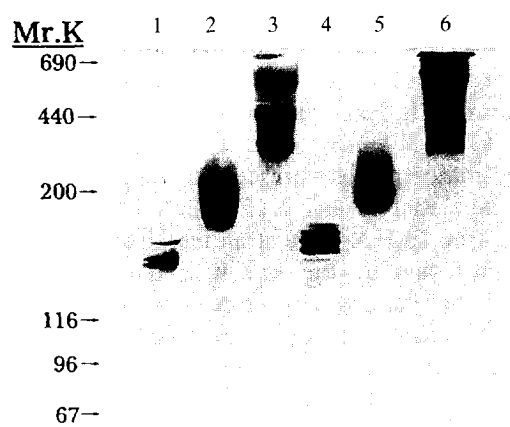

Fig. 5. Analysis of the purity of AIg–mPEG preparations by SDS-PAGE. Samples collected subsequent to purification on anion-exchange HPLC were analyzed on 4–15% polyacrylamide gradient PhastGels (Pharmacia) under non-reducing conditions. First and fourth lanes = un-PEGylated Ig-HA and Ig-V_3C, respectively; second and fifth lanes = Ig-HA–mPEG and Ig-V_3C–mPEG, respectively (content of peak 2 in Fig. 3); and third and sixth lanes = highly derivatized Ig-HA and Ig-V_3C (content of peak 1 in Fig. 3).

between M_r 200 000 and 250 000 (lane 2) were revealed, indicating a mild PEGylation. The degree of PEGylation of these populations was 6–8% according to fluorescamine assay.

The sensitivity of detection of un-PEGylated AIgs corresponds to the limit of detection of silver staining that is approximately 0.05 μg of free AIg. Considering that 5 μg of conjugate were loaded on the SDS-PAGE gels, the preparation contains 1% of free AIgs but 99% of conjugated AIgs.

In summary, we were able to isolate relatively pure populations of AIg–mPEG with 6–8% degree of derivatization as indicated by the electrophoretic and fluorescamine assays. Mildly PEGylated Ig-HA showed a long half life in blood circulation and induced strong T cell activation in vivo. Also mildly PEGylated Ig-V_3C–mPEG was able to elicit anti-V_3C antibody response in mice [26]. Derivatization of the AIgs with mPEG may increase their half life and therefore their efficacy in delivering the viral peptides to the immunocompetent cells.

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