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PEGylation of bacterial cocaine esterase for protection against protease digestion and immunogenicity

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ABSTRACT

Enhancing cocaine metabolism by administration of cocaine esterase (CocE) has been considered as a promising treatment strategy for cocaine overdose and addiction, as CocE is the most efficient native enzyme yet identified for metabolizing the naturally occurring cocaine. A major obstacle to the clinical application of CocE, however, lies in its thermo-instability, rapid degradation by circulating proteases, and potential immunogenicity. PEGylation, namely by modifying a protein or peptide compound via attachment of polyethylene glycol (PEG) chains, has been proven to overcome such problems and was therefore exploited in this CocE investigation.

The PEG-CocE conjugates prepared in this study showed a purity of greater than 93.5%. Attachment of PEG to CocE apparently inhibited the binding of anti-CocE antibodies to the conjugate, as demonstrated by the enzyme-linked immunosorbent assay (ELISA) assay. In addition, PEGylation yielded protection to CocE against thermal degradation and protease digestion. Furthermore, preliminary *in vivo* results suggested that, similarly to native CocE, the PEG-CocE conjugates were able to protect animals from cocaine-induced toxic effects. Overall, this study provides evidence that the PEGylation may serve as a tool to prolong CocE functionality in the circulation and reduce its potential immunogenicity.

1. Introduction

Cocaine is a well known tropane alkaloid acting as a powerful central nervous stimulant and reinforcing drug [1]. Therefore, the abuse of cocaine continues to be a major societal and health problem [2]. Recently, a bacterial cocaine esterase, namely CocE that is found in *Rhodococcus* sp. MB 1 and lives in soil surrounding the coca plant, has been reported to possess a high efficiency in degrading cocaine by hydrolyzing the benzoyl ester of cocaine to produce inactive metabolites, ecgonine methyl ester and benzoic acid [3]. CocE is a globular, 574-amino acid bacterial enzyme with a molecular weight of ~63 kDa, and is among the most efficient protein catalysts characterized to-date for the hydrolysis of cocaine [3]. The major obstacles to clinical application of CocE in treatment of cocaine overdose, however, lie in its temperature-dependent inactivation, with an *in vitro* plasmas half-life of approximately 15 min at physiological temperature (37 °C) [4]. In addition, CocE is prone to enzymatic degradation by circulating proteases. Moreover, since CocE is a bacterial protein that is foreign to the mammalian genome, immunogenicity remains to be a serious concern with regard to its real-time clinical use. While thermo-stable variants of CocE have been developed with a nearly 30-fold increase in plasma half-life observed in both *in vitro* and *in vivo* studies [5], rapid proteolytic degradation and its triggered response by the host immune system remain to be the most critical issues that must be addressed prior to any potential application in clinical practice [6]. PEGylation, in terms of its terminology, means modification of a protein molecule or a carrier cargo (e.g. liposome) by attaching one or several polyethylene glycol (PEG) chains [7]. The PEGylated conjugates normally exhibit several advantages including a reduction or elimination of protein immunogenicity, protection against degradation by metabolic enzymes, and prolongation of the residence time in the circulation [7,8]. Such benefits stem from the steric hindrance effect on the protein molecule by these surrounding bulky PEG chains possessing unparallel special dynamic mobility; a hallmark protection mechanism by PEG. Several examples of success have already been reported. For instance, PEG-asparaginase (Oncaspar®, ENZON Pharmaceuticals Inc., Bridgewater NJ) has been approved by the FDA for clinical use since 1994 in treating acute lymphoblastic leukemia [9]. In addition, PEG-interferon α2a (Pegasys®, Roche, Nutley NJ) by utilizing branched PEG 40 kDa was introduced into the market in 2001 for treating hepatitis C [10,11]. Moreover, branched PEG-anti-

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VEGF aptamer (Pegaptanib, Macugen™, OSI Pharmaceuticals, Melville, NY) has been approved since 2004 for selective management of age-related macular degeneration [12,13].

In this study, conjugation of CocE with both linear and branched PEG_{40K} was investigated. The chemically synthesized PEG-CocE conjugates were purified by passing through an anion-exchange column. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was applied to assess the apparent molecular mass and purity of the PEG-CocE conjugates. *In vitro* assays were carried out to determine the effect of PEGylation on the functionality of CocE in hydrolyzing cocaine. The protective effect of PEG on the *in vitro* thermo-stability and against tryptic digestion of CocE was evaluated at 37 °C. ELISA was employed to evaluate the recognition ability of the PEG-CocE conjugates towards anti-CocE polyclonal antibodies. Lastly, the feasibility of *in vivo* application of PEGylated CocE was examined by using a rodent model.

2. Materials and methods

2.1. Materials

Different types of PEG polymers were obtained from Nektar Therapeutics (San Carlos, CA). Unless otherwise stated, chemicals and reagents were from Fisher Scientific Co. (Pittsburg, PA) or Sigma (St. Louis, MO). Water was distilled and deionized (ddH_2O).

2.2. Methods

2.2.1. Preparation of wild type (WT) CocE and CocE mutants T172R/G173Q

The wild type CocE and CocE mutants T172R/G173Q were prepared according to procedures described previously [5,14]. Point mutations were generated using the Site-Directed Mutagensis Kit (QuickChange, Stratagene, La Jolla CA), and double mutants were generated using single-point mutations as the templates. Wild type and mutant cDNAs were cloned by utilizing the bacterial expression vector, pET-22b (+). All enzymes were expressed as 6× His-tagged proteins in *E. coli* BL-21 (DE3) cells that were grown at 37 °C. Protein expression was induced with 1 mM isopropyl-β-thiogalactopyranoside for 12 h at 18 °C.

Cells were pelleted, resuspended in 50 mM Tris–HCl buffer (pH 8.0) containing 150 mM NaCl, 1 mM dithiothreitol (DTT), and a protease inhibitor cocktail (34 μg/ml each of L-tosylamido-2-phenylethyl chloromethyl ketone, 1-chloro-3-tosylamido-7-amino-2-heptanone and phenylmethylsulfonyl fluoride, as well as 3 μg/ml each of leupeptin and lima bean trypsin inhibitor) and then lysed using a French press (Thermo Fisher Scientific Corp, Needham Heights MA). His-tagged enzymes were enriched 6-fold by using Talon metal affinity chromatography (Clontech Laboratories, Inc, Mountain View CA), and purified using the Q-Sepharose (GE Healthcare, Piscataway NJ) anion-exchange column. CocE was eluted from the Q-Sepharose column with 150–450 mM NaCl linear gradient buffer (pH 8.0) containing 20 mM Hepes, 2 mM $MgCl_2$, 1 mM EDTA, and 1 mM DTT. The peak fractions were pooled, concentrated by using a centrifugal filter device (Carrightwohill, Co. Cork, Ireland), snap frozen in liquid nitrogen, and then stored at −80 °C prior to use.

2.2.2. Preparation of PEG-CocE conjugate via amine group-directed PEGylation

CocE (3 mg/ml) in 50 mM HEPES buffer at pH 8.0 was mixed with mPEG-NHS ester (MW: 5500 Da) at differing [NH_2]:[mPEG] molar ratios ranging from 1:2 to 1:10, based on the number of lysine residues on CocE (8 Lys). Conjugation reaction was performed for 30 min to 1 h at 4 °C in the presence of gentle shaking. The reaction mixture was concentrated and purified by centrifugal filtration (MWCO: 30,000 Da) at 4 °C by adding 10× volumes of cold PBS (50 mM, pH 7.4) four to five times. Prior to and immediately after the conjugation reaction, initial rates of cocaine hydrolysis by the enzyme were measured by monitoring decrease in UV absorbance at 240 nm at 37 °C for 60 s, following the addition of 50 μL 0.5 μg/ml CocE to 950 μL solutions containing a substrate concentration ranging from 7.8 to 125 μM. On an average, approximately 70% of initial enzyme activity was recovered immediately after the PEGylation reaction.

2.2.3. Preparation of PEG-CocE conjugates via sulfhydryl group-directed PEGylation

Prior to the conjugation reaction, CocE was treated with 50 mM DTT for 30 min to break down the disulfide bonds, and the excess DTT was removed by using a Sephadex G-25 desalting column. Solutions containing 4 mg/ml DTT-treated CocE in 50 mM phosphate buffer (pH 7.4) were mixed with the linear PEG_{5K}- or branched PEG_{40K}-maleimide (Jenkem Technology, Allen TX) at a molar ratio of 1:10. The conjugation reaction mixture was stirred for 12 h at 4 °C, and purified with a Q-Sepharose anion-exchange column eluted with 50 mM phosphate buffer (pH 7.4) containing 1 mM EDTA and a linear gradient of 0–500 mM NaCl. Fractions were collected and concentrated by using an ultra-filtration device (MWCO: 10,000 Da).

2.3. MALDI-TOF MS (Matrix-Assisted Laser Desorption/Ionization-Time-Of-Flight Mass Spectra) analysis

The samples were desalted with Pipette Tip (ZipTip, Millipore) by utilizing a saturated solution of sinapinic acid in water/acetonitrile (1:1, v/v) and 0.1% trifluoroacetic acid as the matrix solution. Aliquot containing 1 μL of the sample and matrix mixture was spotted onto a well of the sample plate and dried. Mass calibration was performed using bovine serum albumin and cytochrome *c* as the standards. Mass spectra were taken with linear mode Micromass TofSpec-2E (Waters, Milford MA) equipped with a high mass PAD detector. Data were processed with a MassLynx 3.5 software.

2.4. SDS-PAGE analysis

Fractions obtained under the NaCl gradient were analyzed by gradient (4–20% acrylamide; Invitrogen, Carlsbrad CA) type slab-SDS-PAGE. Gels were stained with coomassie blue, and then analyzed using a densitometer equipped with a digital camera (FE-280, Olympus, Tokyo, Japan) and Image Processing and Analyzing software (ImageJ, National Institutes of Health, Bethesda MD).

2.5. Thermal stability and resistance to trypsin digestion

Thermal inactivation was monitored by measuring the change in cocaine hydrolysis rate. Samples containing 1.6 μM of CocE or PEG-CocE were incubated at 37 °C, and aliquots were taken periodically and subject to enzyme activity assay at 37 °C. The rate of cocaine hydrolysis was determined by calculating the change of the absorbance over a 1-min period at 240 nm using a spectrophotometer (BioTek, Winooski VT). The value attained at each time point was divided by that at the initial time point to obtain the relative value.

The resistance to tryptic digestion was evaluated by mixing trypsin and CocE at a trypsin/CocE ratio of 1:10 (w/w) and incubating with agitation at 37 °C. The samples (CocE or PEG-CocE) were periodically removed to evaluate the cocaine hydrolyzing activity similar to the thermal stability test.

2.6. ELISA assay

The plates were coated with 100 μL of sheep anti-CocE polyclonal serum diluted to 1/2500 in coating buffer, and then the plates were incubated overnight at 4 °C. The liquid in the plate was removed and the plates were washed with PBS-Tween (1 ml Tween in 1 L

phosphate buffered saline (PBS)) solution. The non-specific protein binding sites were blocked by adding the blocking TENTC buffer (pH 8.0) containing 50 mM Tris, 1 mM EDTA, 150 mM NaCl, 0.05% Tween 20, and 0.2% casein. The plates were incubated for 1 h at room temperature. PEG-CocE or CocE solutions (100 μL) were then loaded onto the wells and the plates were incubated for 4 h at room temperature. After washing the plates with the PBS-Tween buffer, an aliquot of 100 μL of rabbit anti-CocE polyclonal serum diluted 1/2500 in TENTC buffer was added into each well and the plates were incubated for 1 h at room temperature. The plates were thoroughly washed with PBS-Tween solution, followed by addition of 100 μL horseradish peroxidase-labeled goat anti-rabbit IgG (KPL, Gaithersburg MD) diluted 1/10,000 in TENTC buffer into each well. The plates were then washed with PBS-Tween solution, and 100 μL of Lumiglow substrate (KPL) were then loaded into each well. Measurements were conducted by recording the absorbance at 430 nm, and data were fitted to a bar graph (Microsoft Excel, Seattle, WA).

2.7. In vivo experiments

Male NIH-Swiss mice (25–32 g) were purchased from Harlan Inc. (Indianapolis, IN). All mice were allowed *ad libitum* access to food and water, and were maintained on a 12-h light–dark cycle with lights on at 6:30 AM to keep at a temperature of 21–22 °C. Experiments were performed in accordance with the Guide for the Care and Use of Laboratory Animals as adopted and promulgated by the National Institutes of Health. The experimental protocols were approved by the University Committee on the Use and Care of Animals at the University of Michigan.

Cocaine-induced toxicity was evaluated by the occurrence of lethality, according to a previously established protocol [4,6]; where lethality was defined as cessation of observed movement and respiration. A preliminary study was performed to determine the ability of PEG_{5K}-CocE to protect cocaine-induced toxicity. Following i.p. administration of several doses of cocaine (320, 560, and 1000 mg/kg; $n = 8$ for each group), mice were immediately placed individually in Plexiglas containers for observation. After 1 min, CocE or PEG_{5K}-CocE (0.3 mg) was administered through the tail vein to each of the animals. Dose–response curves of cocaine-induced lethality were used to compare the *in vivo* protective effect of native CocE versus the PEG-CocE conjugates.

2.8. Statistical analysis

Results are represented as means ± S.D., and data analysis and curve-fitting were conducted by using the Prism 5 (GraphPad Software, San Diego CA) and SPSS 16.0.2 (GraphPad, Chicago, IL). Two-way analysis of variance (ANOVA) was used to test for differences between groups and the level of significance value considered was 0.01.

3. Results and discussion

3.1. Characterization of PEGylated CocE

Initially, PEGylation reaction was performed using different types of PEG derivatives. According to information published in the literature, there were 9 primary amine groups in CocE including those in the 8 lysine residues as well as one at the terminal end of CocE [3]. Monomethoxy-PEG succinimidyl ester (mPEG-NHS; MW 5000 Da) was reacted with these primary amine groups on CocE. Results from MALDI-TOF showed that one to four PEG chains were conjugated to each CocE monomer (Fig. 1). However, PEGylation of CocE via the amine groups was later found to lead a rapid destabilization and loss of the cocaine hydrolyzing activity of the enzyme (data not shown), and this conjugation strategy was therefore abandoned.

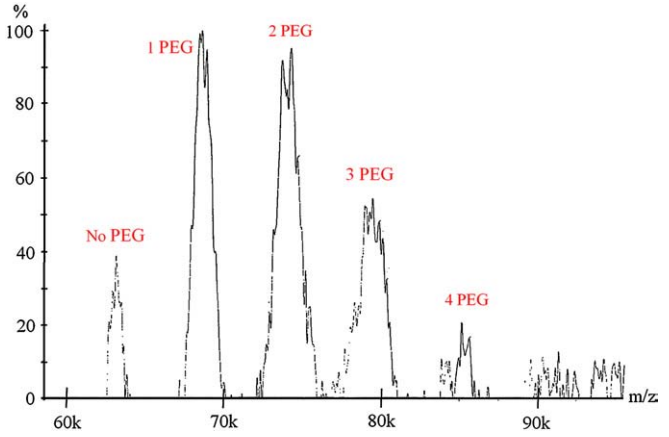

Fig. 1. MALDI-TOF spectra of the PEG-CocE conjugates prepared by coupling PEG succinimidyl ester with the primary amine groups on CocE. Results showed that one to four PEG molecules were linked to each CocE monomer.

PEGylation via the thiol groups was conducted afterwards by utilizing monomethoxy-PEG maleimide (mPEG-MAL; MW 5000 Da). It should be noted PEGylation at thiol groups of cysteine residues not involved in the disulfide bond formation is one of the most specific methods for conjugation [15]. In DTT-treated CocE, there were four free cysteine residues available for conjugation, and two of them were more accessible to PEGylation, based on analysis of the three-dimensional structure of CocE [3]. Hence, PEG-maleimide was used to take advantage of thiol addition to the activated double bond, known as the Michael addition, to yield stable thioether linkages [16]. PEGylation process was performed at the pH 7.0–7.4 to minimize side reaction between the amine group and the maleimido terminal of PEG.

Results from MALDI-TOF showed that one to three PEG chains were conjugated to each CocE monomer (Fig. 2); with two PEG per monomer CocE being the major species. However, further characterization of the properties of these PEG-CocE conjugates revealed the lack of necessary protection of the enzyme's stability, probably due to the fact that CocE was a relatively large molecule and therefore single and short-chain PEG failed to yield sufficient steric stabilization effect on CocE. To this regard, use of larger molecular weight, branched PEG was further explored. It should also be pointed out that using large, branched PEG would help offset the shortage of sites on CocE for PEGylation, as attachment of one branched PEG would be equivalent to the attachment of multiple linear PEG chains.

Following covalent attachment of PEG_{40K}, ion-exchange chromatography using a Q-Sepharose anion-exchange column was applied to separate the PEG-CocE conjugates from unreacted CocE based on differences in charge screening ability between these two species. As

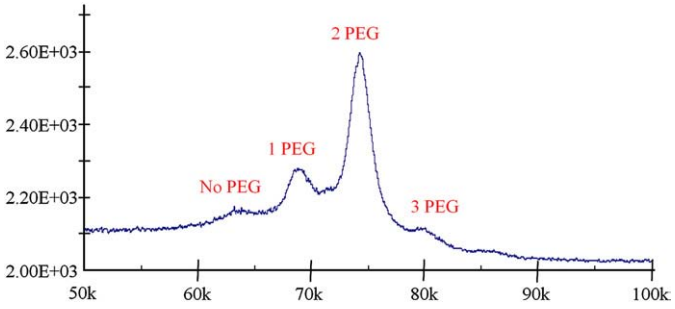

Fig. 2. MALDI-TOF spectra of the PEG-CocE conjugates prepared by coupling PEG maleimide (linear chain, M: 5000 Da) to the thiol groups on the cysteine residues of CocE. One to three PEG molecules were found to be linked to each CocE monomer.

shown in Fig. 3, the PEGylation reaction mixture was separated by Q-Sepharose into five fractions. Fraction #5 was confirmed to contain primarily unreacted CocE, as native CocE conducted on a separate Q-Sepharose chromatography exhibited an almost identical elution retention time as Fraction #5. On the other hand, Fractions #3 and #4 appeared to be PEG-CocE conjugates at a different degree of PEGylation. It is noteworthy that native CocE exists as a dimeric structure (personal communication). Also, according to the MALDI data (Fig. 5, as will be discussed next), no more than one branched PEG_{40K} could be attached to one molecule of cocE. To this regard, Fraction #3 was speculated to be the dimeric CocE with two branched PEG chains (termed PEGylated "homodimer" since there was one PEG per monomeric CocE) since it occurred at a lower salt concentration due to a higher degree of PEGylation thereby yielding a higher degree of charge screening effect towards the Q-Sepharose resin, whereas Fraction #4 seemed to correspond to the dimeric CocE with branched PEG on one of the monomeric unit of CocE dimer (termed PEGylated "hetero-dimer" thereafter). Apparently, Fractions #1 and #2 corresponded to the excess level of PEG polymer and benzoic acid in the conjugation mixture, respectively.

SDS-PAGE analysis provided evidence to confirm this speculation. Unlike ion-exchange chromatography, SDS-PAGE would dissociate the dimeric CocE into monomeric units, thereby rendering it possible to characterize compositions of the PEG-CocE conjugates (i.e. homo- and hetero-dimers) obtained from different fractions in Fig. 3. As displayed in Fig. 4, Fraction #3 seen in the previous figure yielded a major upper band and a minor lower band on SDS-PAGE (i.e. Lane #2). While the upper band corresponded to PEGylated CocE based on the estimated molecular weight, the lower band represented the CocE molecule without PEGylation based on the migration distance observed for native CocE (Lane #4). Based on densiometric evaluation of the gel profile, the purity of the PEGylated CocE seen in the top band was approximately 93.5%; suggesting the presence of primarily PEG-CocE homo-dimers in this fraction. On the other hand, Fraction #4 displayed a mixture of an evenly distributed PEGylated and unreacted CocE monomers (Lane #3), where the bottom band was in almost identical intensity as the top band, implicating the presence of mainly PEG-CocE hetero-dimers in this fraction. MALDI-TOF mass spectroscopy, often employed to identify a purified PEGylated product [16], was used to determine the number of PEG molecules attached to each CocE monomer in Fraction #3 of Fig. 3. As shown in Fig. 5, mass spectra of Fraction #3 yielded a major peak with a m/z ratio of 10,6223 Da, suggesting the presence of one PEG molecule on a CocE; based on its precise correlation to the mass of PEG (40 kDa) and a monomeric CocE (note: a separate MALDI-TOF spectra of native CocE showed a major peak at a m/z ration of 63,338 Da; data not shown). It should be pointed out that, unlike results seen in Fig. 2 in which more than two short 5-kDa PEG chains could be attached to each CocE monomer, only one of the large and branched PEG molecules could be successfully conjugated to each CocE monomer; obviously due to the large hydrodynamic size of the branched PEG. Nevertheless, later studies (see below) of this PEG-CocE conjugate showed that attaching one branched PEG was already effective in yielding steric repulsion against proteolytic degradation and recognition by opsonizing antibodies.

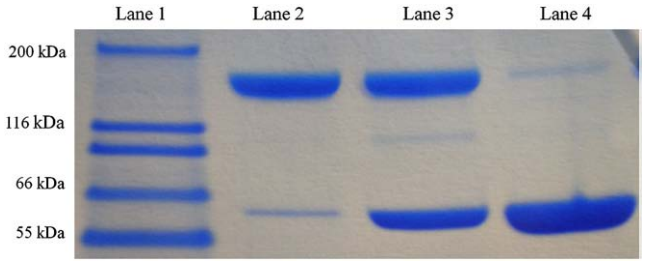

Fig. 4. SDS-PAGE analysis of Fractions #3 and #4 obtained from the elution profile of Fig. 3. Lane 1 showed the molecular weight markers. The top band in Lanes #2 and #3 represented the PEG-CocE monomeric subunit, whereas the bottom band in Lanes #2, #3, and #4 represented CocE monomeric subunit.

A large discrepancy between the calculated molecular weight by the MAlDI-TOF (~105 kDa) method and the SDS-PAGE assay (116–200 kDa) by using the gradient gel was observed. The discrepancy, however, could be accounted for in terms of the difference in the hydrodynamic radii between the compact globular CocE protein and linear PEG polymer [17] and, as a consequence, movement of PEGylated CocE in the SDS-PAGE gel matrix was reduced thereby yielding an apparently larger molecular weight on the calculation [17,18].

3.2. Stability against thermal denaturation and trypsin digestion

After PEGylation, CocE retained approximately 56–75% of its original activity. While the chemical conjugation process undoubtedly would result in a certain degree of irreversible damage on the enzyme, this reduction in biological activity of PEG-CocE could also be attributed to some topological changes occurring in the structure of the enzyme, or to PEG-related steric impediment that could have disturbed protein–receptor interactions and diminished the accessibility of CocE to its substrates or receptor binding sites [19]. When compared with native CocE without PEG protection, Fig. 6 showed that the PEG-CocE conjugates were obviously more stable against thermal denaturation. This could be related to a mechanism reported by other investigators, which suggested that PEGylation could induce a blocking of the intermolecular interactions that were involved in

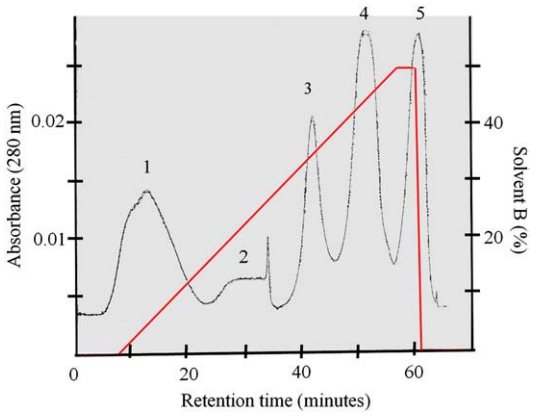

Fig. 3. Ion-exchange chromatography of the reaction mixture of the PEGylation reaction.

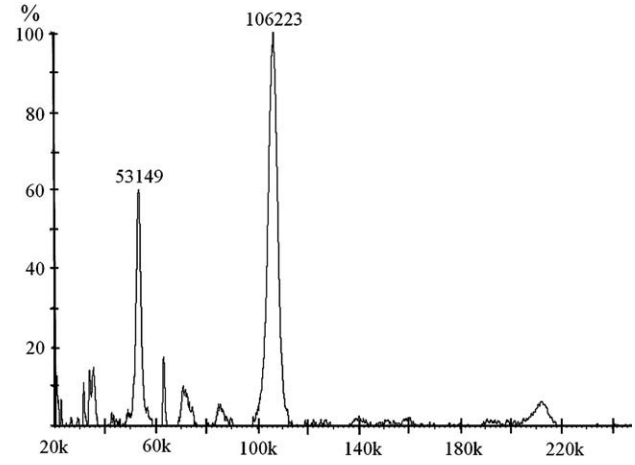

Fig. 5. MALDI-TOF spectra of Fraction #3 obtained from the elution profile of Fig. 3.

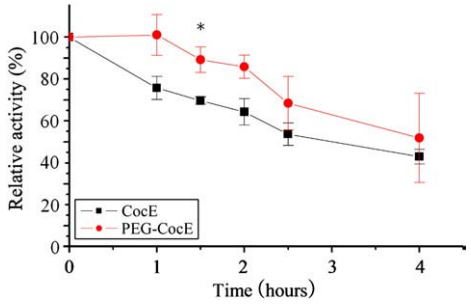

Fig. 6. Time course of thermal inactivation of CocE and PEG-CocE at 37 °C. The *y*-axis represented the ratio of cocaine hydrolyzing activity at a certain time point over the initial activity. *Represented that the difference was statistically significant ($p<0.01$).

thermal instabilities [20,21]. More importantly were the results that the PEG-CocE conjugates exhibited a much stronger resistance to protease digestion than native CocE, as statistically significant difference was observed in the trypsin digestion kinetic profiles (Fig. 7). This finding was somewhat anticipated, as it was demonstrated previously that branched PEG could provide an "umbrella-like" structure, yielding a protection against the approaching and attack by circulating proteases or cells [22,23]. Ample examples of PEG-mediated protection against protease digestion have been reported in the literature, including PEGylated interferon-α, tumor necrosis factor, epidermal growth factor, etc [18,19,24].

3.3. Reduction of immunogenicity by PEGylated CocE

ELISA was conducted to evaluate whether PEGylation could shield the recognition of CocE by specific anti-CocE antibodies. Fig. 8 showed that statistically significant differences in fluorescence intensity between unprotected CocE and PEGylated CocE were observed (2-Way ANOVA, $p<0.01$) over the entire concentration range tested, indicated a decrease in antibody recognition of the PEG-protected CocE. The reduction of 70.4% in antibody recognition is seen with PEG-CocE from the highest concentration when compared with the same concentration of native CocE. Apparently, this reduction in immunoreactivity was attributed to the steric hindrance yielded by the long and branched PEG chains that blocked the interaction between CocE and its specific antibodies; consistent with findings by other investigators on other PEGylated proteins [19]. Also in agreement with literature reports, both the chain length and molecular size of PEG would yield different effects on the protection [7,25]. While the branched, 40-kDa PEG was able to reduce the immunogenicity of CocE, early attempt on the use of the 5-kDa straight chain PEG failed to yield a shielding effect on CocE against anti-CocE recognition (data were not shown); similar to the findings by Caliceti and Veronese [7] on PEGylated urinase, in which branched 10-kDa PEG was found to be far more effective

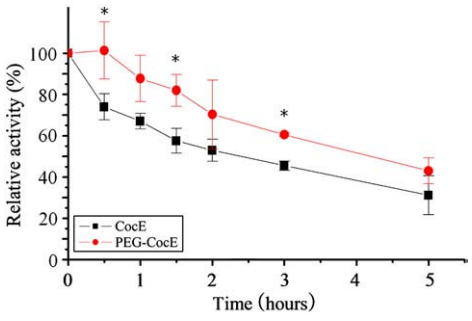

Fig. 7. Resistance to tryptic digestion of CocE and PEG-CocE. PEGylated or unmodified CocE were incubated with trypsin at a trypsin:CocE ratio of 1:10. Reactions were carried out at 37 °C. The PEG-CocE conjugates displayed stronger resistance to typsin digestion than native CocE. *Represented that the statistically significant difference was seen ($p<0.01$).

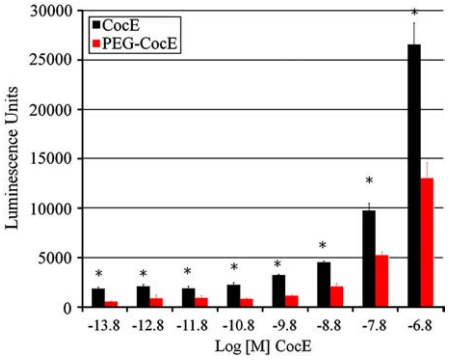

Fig. 8. ELISA assay to assess antibody recognition towards CocE and PEG-CocE. Statistical analysis was performed by utilizing 2-Way ANOVA, and * represented that the difference was statistically significant ($p<0.01$).

than linear 5-kDa PEG in dodging antibody detection. It should be noted that because of the presence of a certain degree (~6%) of unreacted CocE impurity in the obtained PEG-CocE preparation (see above), the observed ELISA results actually represented the minimal degree of PEG-induced protective effects.

Indeed, *in vitro* results correlated very well with *in vivo* findings. It was routinely observed that the effectiveness of CocE therapy gradually declined after repeated administration of CocE to cocaine-challenged mice, with speculation that the development of increased anti-CocE antibody titers had offset the efficacy of CocE during the rescue experiments of cocaine overdose [26]. To this regard, PEGylation could play a crucial role on the success of utilizing CocE in clinical practice in treatment of cocaine abuse or overdose.

3.4. In vivo protection against lethal dose cocaine injection in mice

The previously established rodent model of acute toxicity [4,6] was used to evaluate the effect of PEGylated CocE against cocaine-induced dose-dependent lethality in mice. When PBS solution was intravenously administered into control mice 1 min before intraperitoneal injection of 100 mg/kg lethal dose of cocaine, all (100%) animals died within a short period of less than 15 min. In sharp contrast, when mice were given 0.3 mg of either CocE or PEG-CocE 1 min before i.p. injection of cocaine at a dose 3 times higher than the lethal dose (i.e. 320 mg/kg), all animals were rescued from cocaine-induced lethality. As displayed in Fig. 9, a dose of merely 0.3 mg of CocE or PEG-CocE before cocaine insult already produced a 10-fold shift in the cocaine dose-dependent toxicity profiles. Further increase of the lethal cocaine dose reversed the protective effect by CocE or PEG-CocE in a dose-dependent manner. Indeed, a dose of 1000 mg/kg i.p. cocaine was needed to surmount the protective effect of CocE. It should be pointed out that at an identical dose of 0.3 mg, PEG-CocE exhibited the

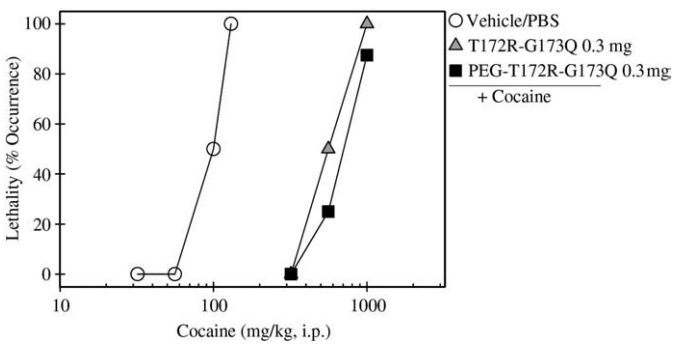

Fig. 9. Protective effects of PEG-CocE against cocaine-induced toxicity. Each data point represents the percentage of mice ($n=8$ for each dosing condition) suffering cocaine-induced lethality.

same potency as native CocE in hydrolyzing cocaine, despite it only consisted of 50% of CocE base on calculation of the protein mass in the PEG-CocE conjugates. This was speculated to attribute to the improved circulating half-life of PEG-CocE due to the steric protection by PEG against proteolytic degradation, although further pharmacokinetic investigation deemed necessary to validate this hypothesis. Nonetheless, this finding corroborated well with several published reports on PEGylated proteins [11,27–31]. For instance, PEGylated α-interferon Pegasys®, which retained only 7% of the antiviral activity of the original protein, still displayed a greatly improved *in vivo* efficacy compared with the unmodified enzyme, primarily due to a markedlyimproved pharmacokinetic behavior [11]. In addition, the *in vitro* ribosome-inactivating activity of PEGylated trichosanthin mutants (PEG_{20K}-Q219C) was found to decrease more than 10-fold yet the *in vivo* activity was increased by 3-fold over the unprotected protein [31]. Furthermore, *in vitro* biological activity of PEGylated growth hormone decreased with increasing the number of attached PEG chains, yet the half-life and *in vivo* potency was reported to increase accordingly [30]. Extensive investigations of the optimal conditions for CocE PEGylation, *in vivo* efficacy against cocaine insult, as well as pharmacokinetics, biodistribution and clearance of the PEG-CocE conjugates are currently in progress in our laboratory.

4. Conclusion

In summary, PEGylation of CocE was successfully carried out and characterized. Both *in vitro* and animal studies provide the proof-of-concept evidence of the feasibility and utility of utilizing PEG protection against thermal denaturation, protease degradation, and clearance by specific opsonizing antibodies. Importantly, our results also showed the practicality of utilizing PEGylated CocE in protecting animals from lethal cocaine insult. Further investigations of the efficacy and safety of this novel treatment modality of cocaine overdose and abuse are currently under way in our laboratory, with the ultimate goal of bringing this project to a successful clinical translation.

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