

CONJUGATION OF TERNATIN BIOMOLECULE WITH POLYETHYLENE GLYCOL ENHANCED CONJUGATES SOLUBILITY AND STABILITY: SYNTHESIS AND PHYSICOCHEMICAL CHARACTERISATION

(Konjugasi diantara Molekul Ternatin dan Polietilena Glikol dengan Penambahbaikan Sifat Keterlarutan dan Kestabilan: Sintesis dan Pencirian Fizikokimia)

Nurul Aina Jamaludin and Noor Faizah Che Harun*

*Green Chemistry and Sustainable Engineering Technology, Polymer Engineering Technology Section,
Universiti Kuala Lumpur, Branch Campus Malaysian Institute of Chemical and Bioengineering Technology,
78000 Alor Gajah, Melaka, Malaysia*

**Corresponding author: noorfaizah@unikl.edu.my*

Received: 15 September 2021; Accepted: 29 October 2021; Published: xx December 2021

Abstract

Ternatin, a highly N-methylated cyclic heptapeptide has promising potential for cancer treatment. However, its efficiency in biological treatment is limited due to its poor solubility and high degradation in aqueous solution. In this present study, methoxy polyethylene glycol (mPEG)-ternatin conjugate abbreviated as mPEG-ternatin was synthesised by direct esterification between a carboxyl end group of methoxy polyethylene glycol, mPEG-COOH and a hydroxyl group in the β -position of ternatin biomolecule which enhanced its solubility and decreased the degradation of ternatin biomolecule in aqueous solution while retaining its inherent anticancer properties. To this end, mPEG-ternatin linked through ester bond was further confirmed using various analytical techniques including FTIR, UV-Vis spectroscopy and HPLC. Importantly, in the solubility and stability studies, the solubility of mPEG-ternatin conjugate was found to be 8% higher than unconjugated ternatin. Furthermore, mPEG-ternatin conjugate exhibited a decreasing percentage of degradation of ternatin biomolecule by 1.9-fold lower than unconjugated ternatin after incubation in 10 mM HEPES pH 7.4 buffer solution at 37 °C for 6 hours. Ultimately, the enhanced properties of ternatin biomolecule by conjugating with a mPEG segment is expected to become new fundamental study in related fields as well as provide new insight into cancer treatments.

Keywords: polyethylene glycol, ternatin, conjugation, esterification, solubility

Abstrak

Ternatin, dengan kandungan N-metil siklik heptapeptida yang tinggi mempunyai potensi untuk rawatan kanser. Walau bagaimanapun, keberkesanannya terbatas dalam rawatan biologi disebabkan rendah keterlarutan dan kestabilannya di dalam air. Dalam kajian ini, konjugat diantara metoksi polietilena glikol (mPEG) dan ternatin, mPEG-ternatin telah disediakan melalui proses pengesteran secara langsung diantara kumpulan karboksil dari metoksi polietilena glikol, mPEG-COOH dan kumpulan hidroksil di posisi β dalam biomolekul ternatin. Hal ini demikian, untuk meningkatkan keterlarutan dan kestabilan molekul ternatin di dalam larutan akues tanpa mengganggu sifat anti kansernya. PEG-ternatin yang dihubungkan melalui ikatan ester disahkan melalui pelbagai teknik analisa yang telah dilakukan iaitu FTIR, spektroskopi UV-Vis dan HPLC. Hasil daripada kajian

ini, keterlarutan biomolekul ternatin di dalam konjugat PEG-ternatin didapati ~8% lebih tinggi daripada molekul ternatin bebas. Sementara itu, melalui ujian kestabilan konjugat, PEG-ternatin menunjukkan penurunan peratusan degradasi molekul Ternatin sebanyak 1.9 kali ganda lebih rendah daripada molekul ternatin bebas. Kesimpulannya, penambahbaikan sifat molekul ternatin dengan kaedah konjugasi secara terus dengan molekul mPEG akan memberikan peluang baru dalam rawatan kanser.

Kata kunci: polietilena glikol, ternatin, konjugat, esterifikasi, keterlarutan, kestabilan

Introduction

In recent years, the research community has been working to create biomimetic materials that mimic the features present in nature. Moreover, a breakthrough has come out in recent development of polymer chemistry using a fundamental theory of polymer phase behaviour at site-specific modification of peptide, synthetic biology, and single-chain polymer [1, 2]. However, there had been slight changes in obtaining successful selective molecular transport, hierarchical structure control, modulated responsiveness to small perturbation, and long-term enzymatic activity [1]. Therefore, researchers had agreed that polymer-biomolecule conjugate would improve this limitation. Mostly polymer-biomolecule conjugates are performed by site-specific, direct conjugation, which allow precise control of the biomolecule to the polymer. Currently, few bioconjugation methods have been discovered, for example, click chemistry, amidation, thiol-maleimide conjugation and esterification [1, 5 - 8]. A biomolecule is chemically conjugated by the conjugation method to form a stable linker or a degradable linker, such as an ester, disulphide, or peptide [9 - 11]. Despite that, esterification is seen as a better alternative due to its simplistic nature, where further modification on biomolecule is not required. Therefore, polymer-biomolecule conjugates formed by direct covalent conjugation via esterification process are a likely-looking new class of materials since one component complements each other.

Ternatin is a highly *N*-methylated cyclic heptapeptide, a natural compound extracted from the mushroom *Coriolus versicolor*. Ternatin biomolecule has been receiving great attention due to its anti-tumour, anti-inflammatory and anti-diabetic properties. Besides, the researchers have discovered that ternatin biomolecule appears to have a fat accumulation inhibitor that can be used to study the structure-activity

relationship (SAR) of the ternatin biomolecule [2 - 5]. Furthermore, Shimokawa et al. revealed that the presence of hydroxyl group located at D-Leu⁷ derivative shows lower inhibition activity than that without this moiety, whereby the presence of the hydroxyl group in the D-Leu⁷ derivative of this moiety is not essential. Therefore, make its availability to install with a new functional group. Moreover, until now, the chemical modifications of the Ternatin biomolecule have been discovered to enhance its biological properties, for example modification of Ternatin biomolecule as molecular probe and biotinylation of Ternatin biomolecule using click chemistry [6, 12, 13]. However, no study has been reported on the conjugation of Ternatin biomolecule with a polymer unit to improve the solubility and stability properties of Ternatin biomolecule.

In polymer-based drug delivery with stealth characteristics, PEG is the most extensively utilised non-ionic hydrophilic and biocompatible polymer [14]. PEG possess an excellent solubility in water as well as in organic solvents making it suitable for the conjugation of polymer-biomolecule. Moreover, the biological inert characteristic of PEG with low toxicity makes PEG perfect for biological applications [14, 15]. Previously, Bayard et al. reported a successful conjugation between mPEG with low molecular weight hydrophobic biomolecules including hormones and antioxidants through esterification leading to excellent drug pharmacokinetic properties [15]. Moreover, there is no fact-finding discussion on the conjugation of Ternatin biomolecule with a polymer unit to the best of our knowledge. Therefore, the remaining challenge inspired this study to develop the direct conjugation of a Ternatin biomolecule with a polymer and investigate its physicochemical properties as well as explore its potential applications.

In the present work, mPEG-Ternatin conjugate was synthesised *via* direct esterification between a carboxyl group of mPEG end group and a hydroxyl group in the D-Leu⁷ derivative of the Ternatin biomolecule using Steglich condition. The reaction was conducted in 10 mM of HEPES with pH 7.4 buffer solution utilising a water soluble carbodiimide with the assistance of HOBT as coupling reagents. The general route employed for the synthesis of mPEG-Ternatin conjugate is shown in Figure 1. The mPEG-Ternatin conjugate was evaluated by their physicochemical properties through high-performance liquid

chromatography (HPLC) analysis, Fourier transfer infrared (FTIR) analysis, and ultraviolet- visible (UV-Vis) spectrophotometer analysis, as well as solubility and stability studies. The results showed that a unit of polymer mPEG was successfully conjugated with a Ternatin biomolecule through esterification, and this conjugated mPEG improved the solubility and stability of Ternatin biomolecule. This new construction of molecular design in this present work is expected to be useful in the related field study such as in bioorganic and medicinal chemistry studies.

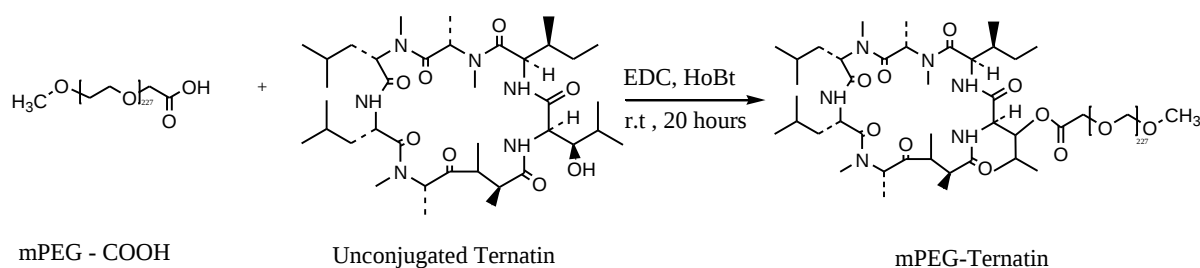


Figure 1. Synthetic design of mPEG-Ternatin

Materials and Methods

Materials

Ternatin was acquired from Cayman Chemical (Michigan) and used as received. Methoxy polyethylene glycol carboxyl (mPEG-COOH) was acquired from Advanced BioChemicals (USA). 1M *N*-(2-Hydroxyethyl) piperazine-*N'*-(2-ethanesulfonic acid) (HEPES), Phosphate buffer saline (PBS), 4-dimethyl aminopyridine (DMAP), 1-Hydroxybenzotriazole hydrate (HOBt), *N*-ethyl-*N'*-(3-dimethyl aminopropyl) carbodiimides (EDC), and ethanol were acquired from Sigma-Aldrich (USA) for the conjugation. Throughout the reaction and the purification process, ultra-pure water obtained from the Milli-Q water purification system was used. All the chemical agents, with an analytical grade were acquired from commercial sources in Malaysia.

Synthesis of methoxy polyethylene glycol and ternatin conjugate

The synthesis of mPEG-Ternatin conjugate was made based on the reported methodology with few modifications [1, 2]. Firstly, 30 mg of mPEG-COOH

was dissolved in 4 mL mixture of 10 mM HEPES with pH 7.4 buffer solution. The mixture was vigorously stirred using a magnetic bar stirrer for 20 hours at 25 °C. To this, 1 mg, and 3 mg of HOBT and EDC were added after fine suspension of PEG were visible. Then, the carboxylate group was activated by continuously stirring the reaction solution for 3 hours. At the same time, the stock solution of Ternatin was prepared in 1 mL of 10 mM HEPES by dissolving 1 mg of Ternatin. Under a nitrogen condition, the mixture of 13.624 μM of Ternatin and 10 mM HEPES was gradually added to the activated PEG while stirring. The mixture was continuously stirred for 20 hours at 25 °C ambient temperature. The final product was purified against 10 mM HEPES, followed by deionised water using an Amicon Ultra-15 Centrifugal Filter Unit (Membrane NMWL= 3KDa) to remove the unreacted molecules. Then, mPEG-Ternatin conjugate was further analysed using various analytical technique including FTIR, UV-Vis Spectroscopy and HPLC.

Physicochemical characterisation: Reverse phase high-performance liquid chromatography analysis

Reverse phase high-performance liquid chromatography (HPLC) using an Ultimate 3000 DIONEX chromatograph equipped with a WPS-3000 autosampler a TCC-3100, a thermostat column compartment, Diode Array Detector DAD-3000, and LGP-3400 RS dual-pump fluid control module, was used to identify the purity of the unconjugated Ternatin and mPEG-Ternatin conjugate (Shelton, USA). The Chromeleon 6.80 software (Dionex Corporation) was used for the data acquisition. RP-HPLC analysis was performed using a reversed-phase C_{18} column as the stationary phase (Brownlee Analytical C_{18} 5 μ m, 150 mm $\times$ 4.6 mm) connected to a guard column (Brownlee Analytical C_{18}) (PerkinElmer Life and Analytical Sciences, Shelton, USA). The analysis of mPEG-Ternatin conjugate using HPLC was carried out following the method from previous literature with some modifications [2, 19, 20]. The mobile phase consisted of two components, A: 0.1% HCOOH in H_2O and B: 0.1% HCOOH in MeCN. The ratio of the components A and B was set at 40:60 (v/v), the flow rate was set at 1 mL/minute, and the total analysis time was 15 minutes. The volume of injection was 10 μ L, the temperature of the sample and column were set at 25 °C ambient temperature, and the detector of UV-visible was set at 200nm and 280 nm to detect the content of unconjugated Ternatin and mPEG-Ternatin conjugate.

UV-Vis analysis

UV-Vis analysis of the emission and absorption spectra of the unconjugated ternatin and mPEG-ternatin conjugate was carried out on a double beam PerkinElmer Lambda EZ210 spectrophotometer using 1 cm length of quartz cuvette in a range of 200 nm to 400 nm to identify the wavelength and absorbance range of the conjugate. The mPEG-Ternatin conjugate was analysed at room temperature, and all the spectra were corrected to the blank data.

Fourier transform infrared spectroscopy analysis

Fourier transform infrared (FTIR) spectroscopy was used to identify the chemical structure characterisation between ternatin biomolecule and mPEG using

PerkinElmer Spectrum RX1. The aim of chemical structure characterisation was to analyse different functional groups that vibrate at a particular frequency. In this experiment, the spectra were obtained by analysing mPEG-ternatin conjugate using FTIR with a range of 250-4000 cm^{-1} and a spectral resolution of 1 cm^{-1} at 25 °C ambient temperature with a single beam purgeable, single compartment.

Solubility analysis

1 mL of PEG-ternatin conjugate was added into 4 mL water and followed by stirring, sonicating and vortexing until the conjugate was fully dispersed in water. Next, the mixture was shaken in a constant shaking incubator for 24 hours before being centrifuged at 14,000 rpm for 5 minutes. The reaction solution was diluted two times lower than initial concentration. The obtained mPEG-ternatin conjugate was calculated from its concentration based on a ternatin calibration plot ($R^2 = 0.996$) by UV-Vis spectrometer at $\lambda = 200$ nm.

Stability analysis

1 mL of mPEG-ternatin conjugate was added into 4 mL of 10 mM HEPES pH 7.4 buffer solution and subsequently, the solutions were incubated at 37 °C for 6 hours. In an hour interval, the sample of mPEG-Ternatin conjugate was taken and the change of absorbance of the conjugate was determined and analysed by UV-Vis Spectrometer.

Results and Discussion

Characterisation of mPEG-ternatin conjugate

The mPEG-ternatin conjugate that was performed in 10 mM HEPES with pH 7.4 buffer solution utilising EDC as coupling reagent with the assistance HOBt at 25 °C for 20 hours was collected and purified by RP-HPLC and centrifugation, respectively. The conjugation percentage was confirmed and identified by HPLC spectra of ternatin absorption wavelength at 280 nm. Figure 2(a) depicts the HPLC spectrum of unconjugated ternatin exhibiting a sharp peak at the 1.55 min of elution time indicating the presence of ternatin biomolecule. Meanwhile, Figure 2(b) indicates the presence of mPEG-ternatin conjugate at 2.3 min of the elution time. The percentage conjugation of mPEG-

ternatin conjugate was calculated based on ternatin biomolecule's standard calibration curve. As a result, the percentage of concentration of mPEG-ternatin conjugate was found to be 49%. This indicates that the direct esterification reaction between the carboxyl group of mPEG, and the hydroxyl group in the β -position of ternatin biomolecule using the Steglich condition was confirmed to be successful and was further confirmed by UV-Vis and FTIR analyses.

Moreover, in order to double confirm the conjugation, UV-Vis study of the emission and absorption spectra was performed from 190 nm until 330 nm for unconjugated ternatin and mPEG-ternatin. The characteristics of the UV-Vis absorption spectrum of unconjugated ternatin and mPEG-ternatin are shown in Figure 3. The peak of UV-Vis absorption of unconjugated ternatin biomolecule was observed at wavelength of 200 nm, whereas after being conjugated, the peak shifted to 280 nm. The emergence of a new absorption peak indicated the covalent conjugation and confirmed the successful conjugation of mPEG-ternatin. The change in the position of these bands

reveals the conformational change in ternatin biomolecule after conjugation with mPEG.

Synthesis of mPEG-ternatin conjugate was achieved by direct esterification of mPEG-COOH and ternatin biomolecule was confirmed by FTIR spectrum as shown in Figure 4. As a result, Figure 4(a) shows the FTIR spectra of unconjugated ternatin in which a broadband at a range of 3000 cm^{-1} to 3500 cm^{-1} was assigned to O-H stretching. Figure 4(b) portrays the FTIR spectrum of mPEG-Ternatin conjugate in which the peak relatively sharper at a range of 3000 cm^{-1} to 3500 cm^{-1} than Figure 4(a) that indicates that the conjugate contains O-H moieties from the ternatin biomolecule. The peak owing to the aliphatic C-H stretching appeared around 2900 cm^{-1} in produced mPEG-ternatin. mPEG-ternatin linked through ester bond is proven using FTIR with the existence of C-O and C=O carboxylate stretching frequencies at 1100 cm^{-1} and 1650 cm^{-1} . The appearance of peaks of ester linkages in FTIR spectra indicated the successful of mPEG-ternatin conjugation.

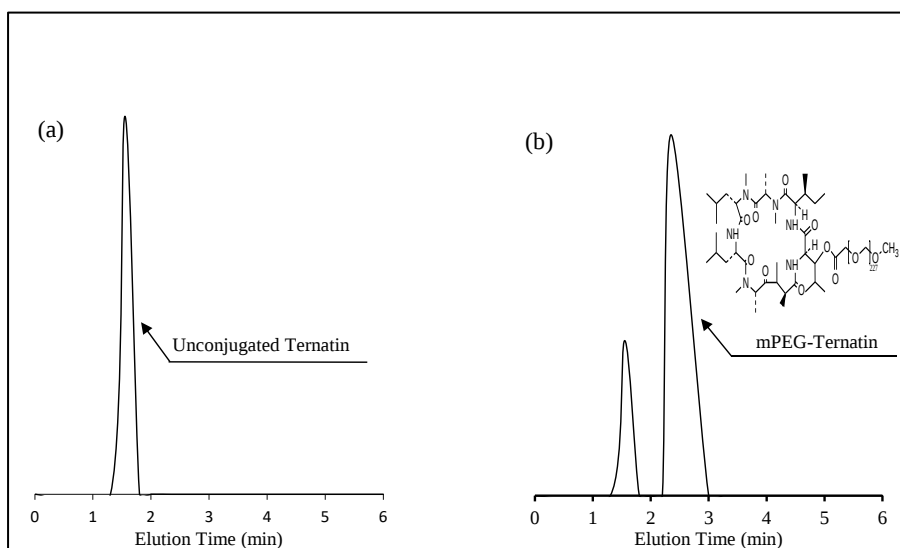


Figure 2. HPLC spectra of (a) unconjugated ternatin and (b) mPEG-ternatin

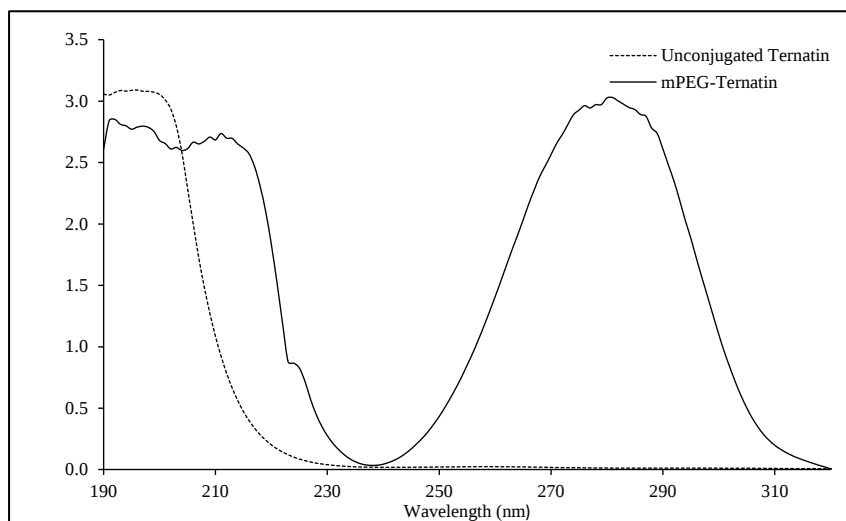


Figure 3. UV-Vis absorption spectrum of unconjugated ternatin and mPEG-ternatin conjugate in 10 mM of HEPES with pH 7.4 buffer solution

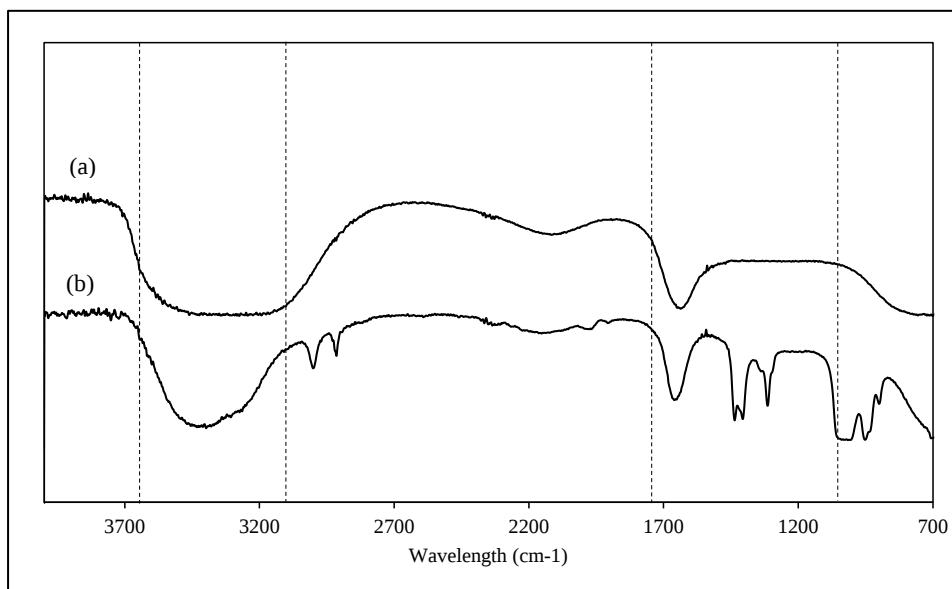


Figure 4. FTIR spectra of (a) unconjugated ternatin and (b) mPEG-ternatin conjugate

The solubility of mPEG-ternatin conjugate

The poor water solubility of Ternatin biomolecule is one of the main obstacles limiting this hydrophobic drug's further medical application. Herein, the solubility of conjugation of ternatin biomolecule with mPEG was explored. PEG conjugation considerably

enhances the solubility of several anticancer drugs, which further improve their bioavailability and antitumor activities. Furthermore, due to the hydrophilic nature of PEG, it forms a hydrophilic outer layer by holding the hydrophobic ternatin biomolecules, which is formed by direct esterification

in aqueous medium. As a result, the solubility of conjugation of mPEG-ternatin was 1.15 $\mu\text{g/mL}$ and unconjugated Ternatin content was calculated to be 1.06 $\mu\text{g/mL}$. This shows that mPEG-ternatin was $\sim 8\%$ higher than that of unconjugated Ternatin due to the strong solubility of PEG. Therefore, the dramatically improved solubility has pushed one step ahead for the medical application of Ternatin biomolecule.

The stability of mPEG-ternatin conjugate

In order to further investigate the ability of mPEG-ternatin conjugate, the stability test was conducted in the mimicking biological environment. Ternatin biomolecule and mPEG-ternatin conjugate stability test was conducted by dissolving in 10 mM HEPES buffer

with pH 7.4 and incubated at 37 °C for 6 hours. An adequate amount of samples was taken every hour, and subsequently, the changes were monitored using UV-Vis spectrophotometer. The percentages of the degradation unconjugated ternatin were calculated based on the initial absorbance of the samples. The result shown in Figure 6 revealed that the percentages of remaining ternatin of mPEG-ternatin conjugate after 6 hours of incubation at 37 °C (61%) was 1.9 times higher compared to the unconjugated ternatin, which is 32%. The higher stability of mPEG-ternatin compared to the unconjugated Ternatin could suggest that the conjugated PEG at the vicinal Ternatin provide a protection to the Ternatin biomolecule in the biological environment.

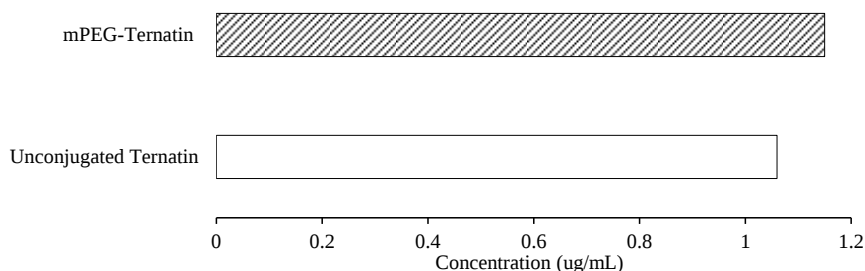


Figure 5. The concentration of unconjugated ternatin and mPEG-ternatin conjugate

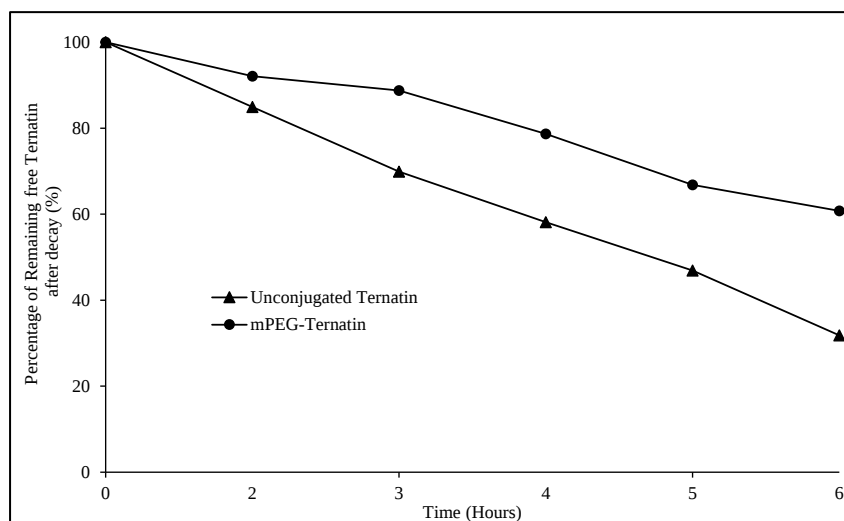


Figure 6. The degradation of unconjugated ternatin and mPEG-ternatin conjugate in 10 mM HEPES buffer pH 7.4 at 37 °C for 6 hours

Conclusion

In summary, mPEG-ternatin conjugate was synthesised by direct esterification between the carboxyl group of methoxy polyethylene glycol, mPEG-COOH and the hydroxyl group at the D-Leu⁷ derivative of the ternatin biomolecule, increasing the aqueous solubility and stability of the ternatin biomolecule relative to unconjugated ternatin. The mPEG-ternatin conjugate significantly improved their solubility, which was ~8% higher than unconjugated Ternatin due to the strong solubility of mPEG. Meanwhile, the stability studies exhibited that mPEG-ternatin conjugate was 29% more stable than unconjugated ternatin due to 1.9 times lower degradation compared to the unconjugated ternatin. Therefore, reported conjugation of mPEG-ternatin conjugate was proven to significantly enhance solubility and stability, thereby promising their future in biological application. Further studies on this topic are now in progress.

Acknowledgement

The authors thank the Ministry of Higher Education (MOHE) Malaysia for the financial support provided via the Fundamental Research Grant Scheme [ref no. FRGS/1/2018/STG05/UNIKL/02/5]. The authors also thank Universiti Kuala Lumpur Malaysian Institute of Chemical and Bioengineering Technology, Melaka, Malaysia for providing resources and necessary facilities for FTIR, HPLC and Light Scattering Intensity analyses. The authors also thank the Nuclear Magnetic Resonance Laboratory, University Industry Research Laboratory, Universiti Teknologi Malaysia, Johor, Malaysia for ¹H NMR analysis.

References

1. Shu, J. Y., Panganiban, B. and Xu, T. (2013). Peptide-polymer conjugates: From fundamental science to application. *Annual Review of Physical Chemistry*, 64(1): 631-657.
2. Dey, S., Ambattu, L. A., Hari, P. R., Rekha, M. R. and Sreenivasan, K. (2015). Glutathione-bearing fluorescent polymer-curcumin conjugate enables simultaneous drug delivery and label-free cellular imaging. *Polymer*, 75: 25-33.
3. Ito, M., Ito, J., Kitazawa, H., Shimamura, K., Fukami, T., Tokita, S., Shimokawa, K., Yamada, K., Kanatani, A. and Uemura, D. (2009). (-)-Ternatin inhibits adipogenesis and lipid metabolism in 3T3-L1 cells. *Peptides*, 30(6): 1074-1081.
4. Shimokawa, K., Yamada, K., Kita, M. and Uemura, D. (2007). Convergent synthesis and in vivo inhibitory effect on fat accumulation of (-)-ternatin, a highly N-methylated cyclic peptide. *Bioorganic and Medicinal Chemistry Letters*, 17(16): 4447-4449.
5. Shimokawa, K., Iwase, Y., Miwa, R., Yamada, K. and Uemura, D. (2008). Whole structure-activity relationships of the fat-accumulation inhibitor (-)-ternatin: Recognition of the importance of each amino acid residue. *Journal of Medicinal Chemistry*, 51(19): 5912-5914.
6. Shimokawa, K., Yamada, K., Ohno, O., Oba, Y. and Uemura, D. (2009). Design, synthesis, and biological evaluation of biotin-labeled (-)-ternatin, a potent fat-accumulation inhibitor against 3T3-L1 adipocytes. *Bioorganic and Medicinal Chemistry Letters*, 19(1): 92-95.
7. Che Harun, N. F., Takemoto, H., Nomoto, T., Tomoda, K., Matsui, M., and Nishiyama, N. (2016). Artificial control of gene silencing activity based on sirna conjugation with polymeric molecule having coil-globule transition behavior. *Bioconjugate Chemistry*, 27(9): 1961-1964.
8. Liechty, W. B., Kryscio, D. R., Slaughter, B. V., and Peppas, N. A. (2010). Polymers for drug delivery systems. *Annual Review of Chemical and Biomolecular Engineering*, 1(1): 149-173.
9. Xu, M., Qian, J., Liu, X., Liu, T. and Wang, H. (2015). Stimuli-responsive PEGylated prodrugs for targeted doxorubicin delivery. *Materials Science Engineering*, 50: 341-347.
10. Böttger, R., Knappe, D., and Hoffmann, R. (2016). Readily adaptable release kinetics of prodrugs using protease-dependent reversible PEGylation. *Journal Control Release*, 230: 88-94.
11. Böttger, R., Knappe, D. and Hoffmann, R. (2018). PEGylated prodrugs of antidiabetic peptides amylin and GLP-1. *J. Control*, 292: 58-66.
12. Shimokawa, K., Iwase, Y., Yamada, K., and Uemura, D. (2008). Synthesis and inhibitory effect on fat accumulation of (-)-ternatin derivatives modified in the β -OH-d-Leu⁷ moiety. *Organic and Biomolecular Chemistry*, 6(1): 58-60.
13. Kawazoe, Y., Tanaka, Y., Omura, S. and Uemura, D. (2014). Design, synthesis, and evaluation, derivatives of the fat-accumulation inhibitor ternatin: toward ternatin molecular probes. *Tetrahedron Letters*, 55(32): 4445-4447.

14. Nair, V., Bang, W. Y., Schreckinger, E., Andarwulan, N. and Cisneros-Zevallos, L. (2015). Protective role of ternatin anthocyanins and quercetin glycosides from butterfly pea (*Clitoria ternatea* Leguminosae) blue flower petals against lipopolysaccharide (LPS)-induced inflammation in macrophage cells. *Journal of Agricultural and Food Chemistry*, 63(28): 6355-6365.
15. Banerjee, S. S., Aher, N., Patil, R. and Khandare, J. (2012). Poly(ethylene glycol)-prodrug conjugates: Concept, design, and applications. *Journal of Drug Delivery*, 2012: 1-17.
16. Knop, K., Hoogenboom, R., Fischer, D. and Schubert, U. S. (2010). Poly(ethylene glycol) in drug delivery: Pros and cons as well as potential alternatives. *Angewandte Chemie - International Edition*, 49: 6288-6308.
17. Bayard, F. J. C., Thielemans, W., Pritchard, D. I., Paine, S. W., Young, S. S., Bäckman, P., Ewing, P., and Bosquillon, C. (2013). Polyethylene glycol-drug ester conjugates for prolonged retention of small inhaled drugs in the lung. *Journal of Controlled Release*, 171(2): 234-240.
18. Gu, Y., Zhong, Y., Meng, F., Cheng, R., Deng, C. and Zhong, Z. (2013). Acetal-linked paclitaxel prodrug micellar nanoparticles as a versatile and potent platform for cancer therapy. *Biomacromolecules*, 14(8): 2772-2780.
19. Stompor, M., Switalska, M., Bajek, A., and Wietrzyk, J. (2019). Influence of amide versus ester linkages on the anticancer properties of the new flavone-biotin conjugates. *Zeitschrift Fur Naturforschung - Section C Journal of Biosciences*, 74(7-8): 193-200.
20. Iwakiri, T., Mase, S., Murakami, T., Matsumoto, M., Hamada, H., Nakayama, T. and Ozaki, S. I. (2013). Glucosylation of hydroxyflavones by glucosyltransferases from *Phytolacca americana*. *Journal of Molecular Catalysis B: Enzymatic*, 90: 61-65.