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Physico-Chemical Characterization of Polyethylene glycol-Conjugated Betulinic Acid

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Abstract. Betulinic acid (BA) is a naturally occurring plant pentacyclic triterpenoid with activity against cancer and infectious diseases like malaria and AIDS. Its pharmacological activity is limited by low aqueous solubility and bioavailability. Attempts have been made to improve the solubility of BA by conjugation to the water-soluble polymer polyethylene glycol (PEG) but with very limited physico-chemical characterizations. This work presents physico-chemical characterizations of a PEG-BA conjugate using ^{1}H NMR spectroscopy, electron microscopy, DLS and XRD. The NMR data showed successful conjugation through the formation of an amide bond with a 5% drug loading although the appearance of some chemical shift signals were solvent-dependent. TEM images showed a spherical morphology of the conjugate with average diameter of 59.58±4.47 nm.

INTRODUCTION

It is estimated that over 80% of the world's population relies on herbal medicines as their primary health and treatment option (1). Herbal medicines have also been the source of many clinically-approved drugs and have acted as scaffolds or templates for synthetic derivatives. Artemisinin and its derivatives (artemether, dihydroartemisinin and artesunate) are example of plant-extracted parent compound. The success of these drugs has inspired the search for other pharmacologically active compounds from plants (2,3). Betulinic acid (BA) (**Fig. 1**) is a pentacylic triterpenoid obtainable from the shrub, *Ziziphus nummularia*. BA has a wide spectrum of pharmacological activities which include antimalarial, anti-HIV, anti-cancer, hepatoprotection, anti-depression and antioxidant. The main pharmacological limitations of BA are its limited aqueous solubility, poor membrane permeability and low bioavailability (4). Attempts have been made to improve the pharmacological potentials of the molecule especially for cancer treatment. One strategy has involved the covalent linkage of BA to a polymeric carrier Dai et al. (5) and Saneja et al. (3). Dai et al. (5) used a multiarm polyethylene glycol (PEG) to conjugate BA for the treatment of lung cancer and the results showed prolonged circulation of the BA in the blood and with enhanced tumor targeting. Saneja et al. (3) also reported the PEGylation of BA for the treatment of liver cancer that showed improved solubility, stability and enhanced cellular internalization.

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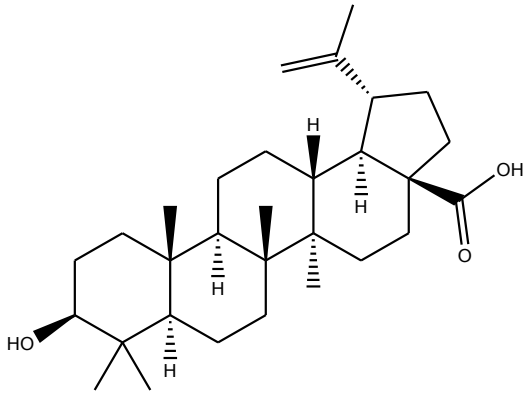


FIGURE 1. Chemical structure of betulinic acid

PEG is a synthetic polymer of ethylene glycol that is well-established in pharmacological delivery systems. It is biocompatible, amphiphilic and possesses high hydration capacity (6-8). It is electrically neutral at all pH ranges with highly active functional terminals (**Fig. 2**). The two terminal hydroxyl functional groups can be derivatized into a wide selection of functional groups like amines, carboxylic acids, thiols, ethers etc (9). It is biologically inert, non-immunogenic and exhibits poor plasma protein adsorption. It is often used as a long circulating drug carrier due to its poor tissue uptake (10).

BA was conjugated to PEG through a physiologically reversible bond like an ester or amide and the resulting polymer-drug conjugate is a pharmacologically inactive prodrug (3,5). As a new chemical entity (NCE), PEGylated BA has physico-chemical properties dictated by the polymer. Previous reports of PEG-BA conjugates have mainly focused on the biological performance of the NCE construct (3,5). It is important that as with any other pharmaceutical agent a detailed characterization be conducted to allow for a full understanding of its properties and performance (11). We report on further physico-chemical characterizations of a PEG-BA conjugate which have not been previously presented in the research literature. Our preliminary study of its pancreatic cancer inhibitory activity is promising.

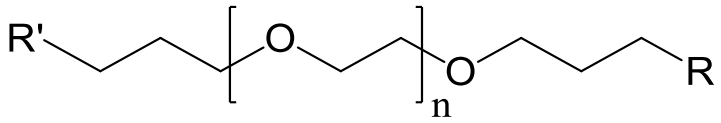


FIGURE 2. Chemical structure of PEG where R and R' represent a varied selection of terminal functional groups (12).

EXPERIMENTAL

Reagents and Materials

Poly (ethylene glycol) bis(3-aminopropyl) terminated MW=1500 Da, dimethylformamide (DMF), dichloromethane (DCM), deuterated pyridine, 4-(dimethyl amino) pyridine (DMAP) deuterated dimethyl sulfoxide (DMSO), deuterated water (D_2O), ethyl acetate (EtOAc), hexane, betulinic acid, sulfo N-hydroxy-succinimide (sulfo-NHS), and phosphotungstic acid hydrate were purchased from Sigma-Aldrich. All chemicals were of reagent-grade and used as received. Pur-A-lyzer Mega 1000 dialysis kit was also purchased from Sigma-Aldrich. Lyophilization was carried out with a Telstar LyoAlfra10 freeze-dryer (Spain) at -60 °C at 0.3 mBar.

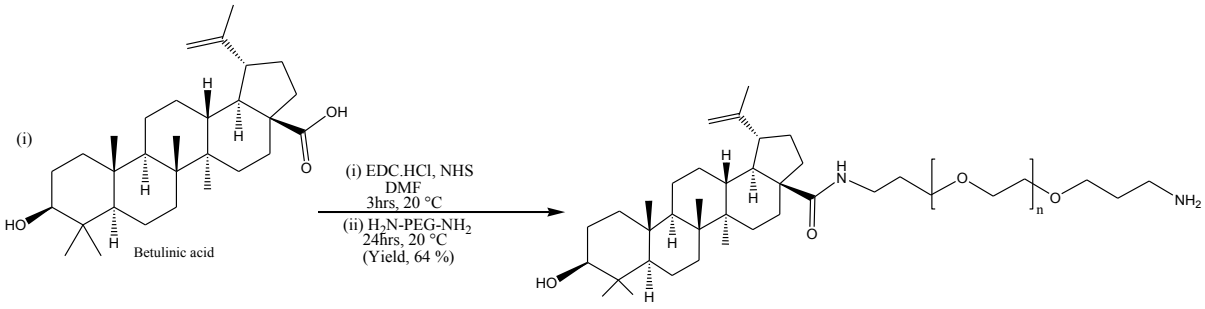
Synthesis of PEG-BA Conjugate

PEG-BA conjugate was prepared using a two-step synthesis method previously described by Saneja et al. (3) and Dai et al. (5) but with modifications. Briefly, BA (6.09 mg, 1 mol *equiv*) was activated using EDC (5 mol *equiv*.) and NHS (5 mol *equiv*.) in DMF (2.0 mL) and the reaction was allowed to proceed for 2 hrs at 20 °C. Then, without product

isolation, the activated BA solution was added dropwise to PEG (MW 1500 Da, 20 mg, 1 mol *equiv*) dissolved in DMF (1.0 mL) with vigorous stirring. DMAP was added as a catalyst. The reaction was allowed to proceed at 20 °C for 24 hrs. The solvent was concentrated *in vacuo* and the crude product was dissolved in THF (1.0 mL). The PEG-conjugated BA was precipitated with cold diethyl ether (5.0 mL) to yield the pure PEG-BA conjugate at 64% recovery yield with R_f = 0.13 (hexane : ethyl acetate, 1 : 3). The final product was dissolved in PBS buffer (pH = 7.40) and lyophilized.

RESULTS AND DISCUSSION

Synthesis of PEG-BA and Characterization



SCHEME 1. The synthesis sequence of the PEG-BA conjugate.

The conjugation of PEG to BA via carbodiimide chemistry resulted in a 64% yield of the product [**Scheme 1**]. Conjugation was confirmed by ^{1}H NMR (400 MHz in deuterated pyridine) (**Fig. 3**). The ^{1}H NMR spectrum of BA in **Fig. 3** (a) exhibited a multiplet from 0.79 ppm to 2.69 ppm due to the terpenoid protons.

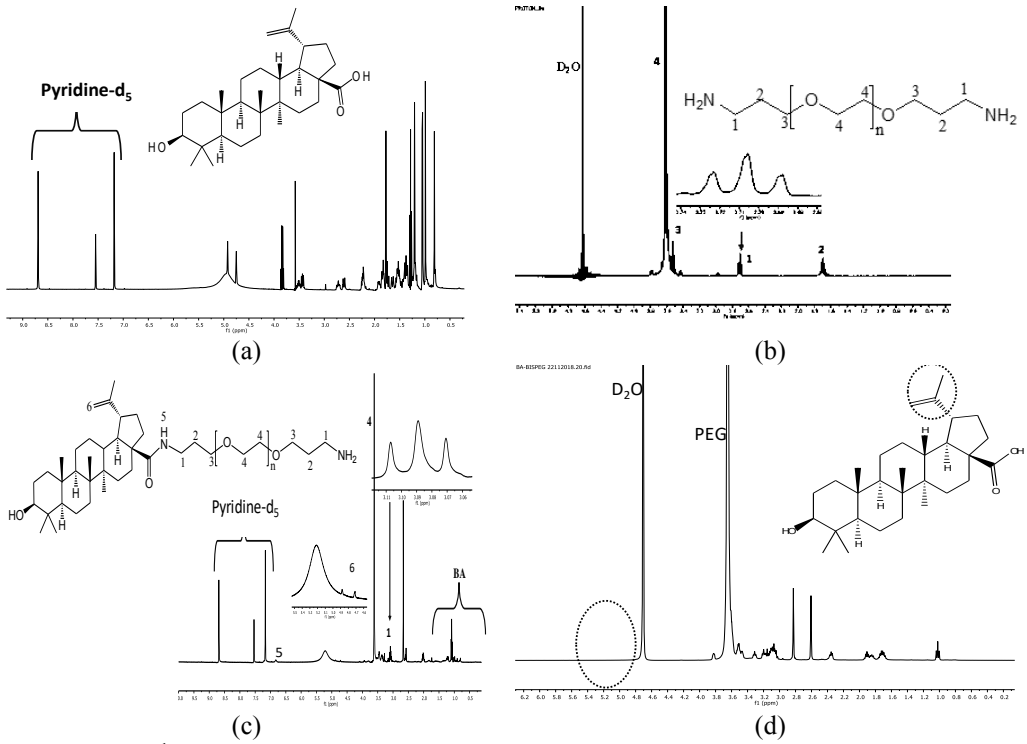


FIGURE 3. The ^{1}H NMR (400 MHz) spectra of (a) betulinic acid (BA) in pyridine-d_5, (b) PEG in D_2O, (c) PEG-BA conjugate in pyridine-d_5 and (d) PEG-BA conjugate in D_2O

The signals at 4.90 ppm and 4.74 ppm are attributed to the alkenyl terminal protons. In **Fig. 3 (b)**, PEG presented signals at 3.4-3.6 ppm for the protons of -O-CH_2-CH_2- and the methylene protons of CH_2-NH_2 appear at 2.69-2.72 ppm when D_2O is used as solvent. The ^{1}H NMR spectrum of the PEG-BA in **Fig. 3 (c)** in pyridine-d_5 showed signals of both BA and PEG, with the appearance of the amide peak signal at 6.83 ppm. In D_2O, the PEG-BA conjugate does not show some of the characteristic peaks of the BA around 4.7-4.9, which are attributable to alkene (**Fig. 3 (d)**). Saneja et al. had conducted the NMR experiments in DMSO-d_6 even though the PEG-BA is soluble in water (3). The loss of crucial proton signals in D_2O shows why the solvent is not suitable for ^{1}H NMR analysis. A postulated cause of the signal loss could be the folding and encapsulation of the drug in D_2O as the conjugates assume a more thermodynamically stable micellar aggregation in the aqueous environment compared to when an organic solvent is used. The percentage drug loading was determined from the integration of BA and PEG peaks in the ^{1}H NMR spectrum to be 5%.

Morphology, Particle Size and Particle Size Distribution

The morphology of the PEG-BA conjugate was studied using transmission electron microscope (TEM) (**Fig. 4 (a)**). TEM micrographs reveal that the nanoparticles are spherical in shape and monodispersed with an average size diameter of 59.58±2.36 nm (**Fig. 4 (b)**)**.** The morphology of the nanoparticles in aqueous media supports the postulation that in D_2O the PEG-BA conjugate aggregates into an inverted micellar structure (**Fig. 4 (c)**), where the hydrophobic BA head of the conjugate is encapsulated in a spherical structure while the hydrophilic PEG tail forms the solubilizing corona.

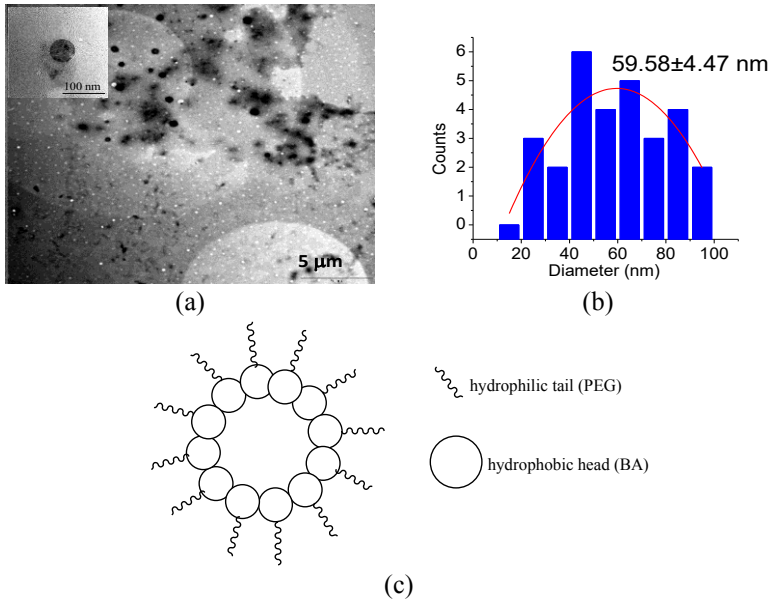


FIGURE 4. (a) TEM image and (b) Particle size distribution histogram and (c) representation of the inverted micellar structure.

Table 1 presents the mean particle size distribution and poly-dispersity index (PDI) of the PEG-BA conjugate. The conjugate had a hydrodynamic size of 125.7±2.30 nm with a PDI of 0.225±0.007. A PDI value closer to 1 is an indication of a broad size distribution (13).

TABLE 1. Dynamic light scattering of PEG-BA

Parameters tested	PEG-BA
Particle size distribution (d.nm)	125.7±2.30
PDI	0.225±0.007

X-ray diffraction (XRD) was also used to study the physical state of the PEG-BA shown in **Fig. 5**. The XRD analysis in **Fig. 5 (a)** showed a crystalline nature of the drug (BA). The PEG exhibited two peaks at 2θ = 19.3° and 23.4° in **Fig. 5 (b),** indicating that the polymer is also crystalline in nature. The PEG-BA conjugate showed peaks of the PEG with the appearance of new peaks at 2θ = 32.5° and 47.1° indicated by a star in **Fig. 5 (c).** The appearance of new peaks suggest change in the physical structure due to conjugation. However, the appearance of the unchanged polymer peaks suggests that the bulk structure of the polymer component of the conjugate did not change.

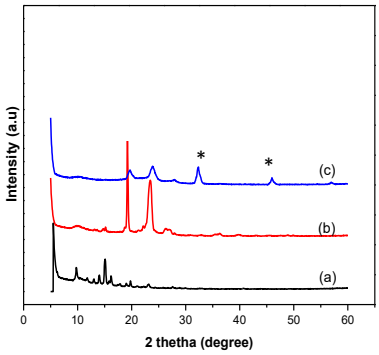


FIGURE 5. X-ray diffraction of (a) BA, (b) PEG and (c) PEG-BA conjugate

CONCLUSION

The NMR experiments of PEG-BA showed that careful consideration of deuterated solvent selection is critical for full signal identification. Owing to its amphiphilic structure, PEG-BA appears to aggregate into an inverted micellar conformation in an aqueous environment as observed by TEM and with average hydrodynamic particle size of 125.7±2.30 nm. Such size and conformation enhance aqueous solubility and cellular uptake of BA. XRD also suggested a change in the physical structure upon conjugation even though the bulk structure of the polymer was unaffected.

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