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DESIGN AND SYNTHESIS OF NEW POLYETHYLENE GLYCOL-HYALURONIC ACID-MELPHALAN (PEG-HA-MEL) AS A TARGETED ANTITUMOR DRUG

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ABSTRACT

Background and aims: Melphalan (MEL) is a non-target selective anticancer agent with a short plasma half-life. The objective of the current study was to design targeted delivery and extend plasma half-life of MEL so as to enable it to play a better antitumor role. **Methods:** Polyethylene glycol-Hyaluronic acid (PEG- HA) was obtained by modifying Hyaluronic acid (HA) with activated PEG. PEG-HA was then connected with MEL to prepare the PEG-HA-MEL prodrug. The prodrug generates micelles by self-assembly in aqueous solution, and was characterized by nuclear magnetic resonance and infrared spectroscopy. Changes of tumor cell morphology and cytotoxicity were observed by an inverted microscope. **Results:** In this study, the PEG-HA-MEL prodrug was synthesized by PEG-HA connected with MEL. Dynamic light scattering (DLS) and transmission electron microscope (TEM) showed that the prodrug formed micelle and was evenly distributed in 100-130nm. Tumor cells treated with PEG-HA-MEL (10^{-6} mol/L) were condensed and rounded, aggregated into clumps, and the number of cells was significantly reduced at 24 hr and 48 hr after treatment, respectively. **Conclusion:** By connecting PEG-HA with MEL, it was found that this compound played a potent inhibitory role on tumor cells. This is a new method for the synthesis of PEG-HA-MEL prodrugs as targeted antitumor therapy.

KEYWORDS: Melphalan, Polyethylene glycol - Hyaluronic acid, Hyaluronic acid, Synthesis, Antitumor.

INTRODUCTION

Cancer is a complicated disease representing one of the leading causes of mortality in the world. The greatest challenge in developing anti-cancer drugs is delivering them to the target tumor tissues, and reducing systemic toxicity. Due to the constant progress accomplished in the fields of medicinal chemistry, nanotechnology and in the understanding of the biological mechanisms of cancer diseases, some drug delivery approaches have been developed to enhance target delivery and to improve efficacy of existing anti-tumour drugs.[1] The "Prodrug" strategy was one of those strategies, designed many years ago to help drugs cross physiological barriers. In this approach, the prodrug consists of an active drug linked to a carrier that will help it to reach the pharmacological target, and then it is ensured that the carrier can be removed afterwards and the biologically active compound regenerated.[2]

Melphalan (MEL) is an alkylating agent, which inhibits DNA synthesis. It has been proven effective against ovarian, breast and colon cancers.[3] However, it has some obvious drawbacks such as short plasma half-life, non-target selectivity, and serious adverse reactions. Because of these shortcomings, its clinical application is limited.

Hyaluronic acid (HA) is a naturally occurring linear polysaccharide. HA receptors such as homing cell adhesion molecule (CD44) and Hyaluronic acid-mediated cell migration receptor (RHAMM) are abundantly presented in tumor cells.[4] Tumor cells show enhanced binding and internalization of HA. Furthermore, it has been shown that an active drug linked to HA specifically binds with tumor cells after entering the body, followed by intracellular release of active drugs, thus restoring their original cytotoxicity.[5-7]

Polyethylene glycol (PEG) is a small nontoxic linear molecule, which has been extensively investigated for the development of tumor-targetable prodrugs, due to its capability of keeping the prodrug in circulation for a long duration (extend plasma half-life of MEL), followed by their passive accumulation in the tumor tissue.[8] Particularly, the PEG surface can enable prodrugs to escape from the reticuloendothelial system, thus minimizing their removal at the liver site. PEG has been used to modify biomedical materials such as proteins, polypeptides, enzymes and biochemical drugs.

The activated PEG was chosen to couple with HA at a high-speed in mild conditions, in which reaction that the carboxyl end of activated PEG react with the amino acid end of acetylated HA to get binary conjugate content of an ester chain. Therefore the amino tail of MEL reacts with the carboxyl tail of HA to form PEG-HA-MEL (Figure 1).

MATERIALS AND METHODS

Chemistry and instrumentation

N-Hydroxy succinimide (NHS) and 1-Ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC) were purchased from J & K Chemical. MEL and HA (Mw=1050kDa) were obtained from Wuhan Hezhong Biochemical Manufacturing Co. LTD. Succinic anhydride was purchased from Tianjin Kaitong Chemical Reagent Co. PEG-4-dim ethyl amino pyridine (DMAP) was obtained from Wuhan Chemical Reagent Co., LTD.

The FT-IR spectra were recorded on pressed KBr pellets using a Nexus Fourier infrared spectrometer (Therma Nicolet spectrometer, USA) at room temperature in the range between 4000 and 500 cm^{-1}.

Bruker avance-400 (Brucker, Switzerland) was used to acquire ^{1}HNMR spectra and a JEM-2010HT transmission electron microscope (Japan Electronics and Oxford Compan, Japan) was employed to get the sample morphology, dispersion degree and particle size information.

General procedure for preparation of PEG-active ester

With pyridine (2ml) as a catalyst, a solution of PEG-2000 (10g) and succinic anhydride (2.5g) in chloroform (100ml) was heated for 6 hours then evaporated until a viscous liquid was obtained. After cooling in the ice bath, anhydrous diethyl ether was added to the solution. The produced (PEG-SA) precipitate was filtered and recrystallized in chloroform and anhydrous diethyl ether.[8,9]

The mixture of PEG–SA、NHS and EDC were dissolved in appropriate amount of N, N-dimethylformamide (DMF), and reacted overnight by constant magnetic stirring.[10] After that, anhydrous diethyl ether was added to the solution in drops, stirred in ice bath for a few minutes, until no more precipitates were generated. The precipitate was filtered and then dissolved in chloroform and recrystallized in anhydrous diethyl ether.

Figure 2A showed the FTTR spectra of PEG-active ester, which is a white solid, with 70% yield. The absorption band at 3481cm^{-1} was the characteristic peak of a hydroxyl, the band at 2955cm^{-1} was attributed to the hydrocarbon telescopic vibration of a methyl-CH_2, which belonged to a PEG characteristic peak. The band at 2877cm^{-1} was telescopic vibration of -N; which was the characteristic peak of NHS. The band at 1712cm^{-1} was carbonyl telescopic vibration of an ester, the peak at 1120cm^{-1} was telescopic vibration of C-O. The result showed the compound contained the ester bond, which is PEG active ester, was synthesized successfully.

The ^{1}H NMR spectra of PEG-active ester was shown in Figure 2B. The signals at A (1.50 ppm) was attributed to the methyl protons in succinic anhydride**.** Successful conjugation of PEG was confirmed by the peak appearing at B (3.6 ppm [-CH_2-CH_2-O-]) (Figure 2C).

General procedure for preparation of PEGylated HA

HA (0.5g) was dissolved in NaOH (20%, 15ml) solution, after magnetic stirring until the colloidal material gradually turned into uniform solution. Then the PEG-active ester dissolved in water was added, reacted for 6 hours at room temperature.[8,11] Flocs were obtained by adding ethanol (95%) to the solution, then filtered and washed with ethanol 3 times**.** A pale yellow solid was obtained, namely PEGylated-HA (PEG-HA).[12,13]

Figure 3A showed the FTIR spectra of HA and PEG-HA, which is a light yellow solid, with 78% yield. The bands at 3425 cm^{-1} and 3431cm^{-1} were characteristic peaks of hydroxyl, the peak at 2921 cm^{-1} was the hydrocarbon telescopic vibration of -CH2-.The bands at 1626 cm^{-1} and 1572 cm^{-1} were characteristic peaks of amide. Likewise, the peaks at 1577cm $^{-1}$ and 1618 cm^{-1} attributed to amide characteristic peaks of PEG-HA, while the band at 1449 cm^{-1} was bending vibration of modified polyethylene glycol part. Finally, the band at 1384cm^{-1} was bending vibration of methyl hydrocarbon of HA amide. The results showed that: HA was successfully connected to the PEG, because there are characteristic absorption peaks of modified PEG and amide bond in PEG-HA.

The^{1} H-NMR spectra of HA and PEG-HA were shown in Figure 3B. The signals at C (2.00pm) and D (3.75-3.20ppm) were attributed to the methyl protons in acetyamine groups and the protons in the sugar ring of HA, respectively. The signals at F (3.2-2.20ppm) was attributed to the methylene protons of PEG. The characteristic peaks at E (3.60ppm) corresponded to the methylene in the α position of the ester groups.

General procedure for preparation of PEG-HA-MEL Micelles

PEG-HA (300mg), EDC (260mg) and DMAP (0.05mmol) were dissolved in anhydrous DMSO (15ml) while avoiding light and stirred at room temperature till PEG-HA was dissolved completely.[14] MEL (10mg) and NHS (0.05mmol) were added, the mixture was allowed to react without light for 24 hr. Then the solution obtained was dialyzed to form micelle solution. After dialysis and freeze-drying, the product was named PEG-HA-MEL (prodrug).

Figure 4A showed the FTTR spectra of MEL, PEG-HA and PEG-HA-MEL, which is light yellow solid, with

56% yield. As shown, the peaks between 3500 cm^{-1} to 3400cm^{-1} may be characteristic peaks of hydroxyl, amino or hydrogen of benzene rings. The bands at 1600cm^{-1} and 1599cm^{-1} were characteristic peaks of an amide and a carbonyl, while the peak at 1100cm^{-1} was stretching vibration of C-O. Absorption peaks of benzene were at around 1600cm^{-1} to 1400cm^{-1}.

The product of PEG-HA-MEL was placed into an analysis bag, dialyzed with 1.5 L deionized water until all small molecules passed through, and micellar solution was obtained by self-assembly in aqueous solution.[15, 16]

However, micelles are not suitable for storage in aqueous solution. Therefore, the solution in the dialysis bag is distilled under reduced pressure at normal temperature, and the solvent of DMSO was removed.[17,18] It was then diluted with double distilled water to 50mL, filtered with microporous filter membrane, and freeze-dried to obtain PEG-HA-MEL lyophilized powder.

Figure 4B showed the ^{1}H-NMR spectrum of the micellar solution of PEG-HA-MEL. It can be seen that there was no peak at 7.0ppm in D_2O with the characteristic peaks of benzene. This was because PEG-HA-MEL was an amphiphpilic polymer. It self-assembled in water to form a core-shell structure. MEL on the other hand is lipophilic and the observed hydrophilic property of PEG was strong in the process of self-assembled formation of PEG-water-based shell in which MEL was wrapped. So it did not respond in the ^{1}H-NMR spectrum graph.

Cell culture and cytotoxicity assay

SKOV3 tumor cells (China Type Culture Collection. Wuhan, China) were maintained at 37^0C in a humidified incubator with 5% CO_2/95% air and propagated in Dulbecco's Modified Eagle Medium containing 100 mg/dl d-glucose, 10% fetal calf serum, 100 U/ml penicillin, 100 µg/ml streptomycin, and 2 mmol/l supplemental glutamine. Cell lines were passaged once per week after treatment with trypsin-ethylenediaminetetraacetic acid. Cells were used for experiments from *passages 5–15*. Assay was carried out by seeding 3000 SKOV3 tumor cells in each well of 96-well culture plates. During this final incubation, $SKOV_3$ tumor cells were treated with one of the following: (1) control; (2) MEL (10^{-6}mol/L); (3) PEG-HA-MEL (10^{-6}mol/L) for 0 hr, 24 hr, and 48 hr. Changes of tumor cell morphology were observed respectively by inverted microscope at 0 hr, 24 hr, and 48 hr.

RESULTS

Synthesis

The synthetic route and its optimal conditions of PEG-HA-MEL modified hyaluronic acid with activated PEG has been determined in this paper. Then cross-linked hyaluronic acid (PEG-HA) was then connected to MEL. The prodrug PEG-HA-MEL generates micelles by self-assembly in aqueous solution. Therefore, chemical modification of MEL was achieved to improve the drug efficacy in tumor cells. The compounds were characterized by nuclear magnetic resonance and infrared spectroscopy.

DLS characterization of PEG-HA-MEL

Particle size distribution of PEG-HA-MEL was determined by DLS.PEG-HA-MEL size distribution was shown in Figure 5A. The figure showed the size of nano particles was evenly distributed in between 100~130nm and most of them were about 116.4nm.

TEM characterization of PEG-HA-MEL

PEG-HA-MEL was dissolved in DMSO, and sample morphology, dispersion degree and particle size information through TEM were obtained. From Figure 5B, it was found that the particle size was around 120nm, and the morphology of micelles is spherical, which was consistent with that of DLS.

Cell cytotoxicity and morphological analysis

Figure 6 showed that the control cells demonstrated normal growth at 0 hr, 24 hr, and 48 hr. The cells treated with MEL (10^{-6} mol/L) and PEG-HA-MEL (10^{-6} mol/L) were condensed and rounded, aggregated into clumps, and the number of cells was significantly reduced at 24 hr and 48 hr respectively.

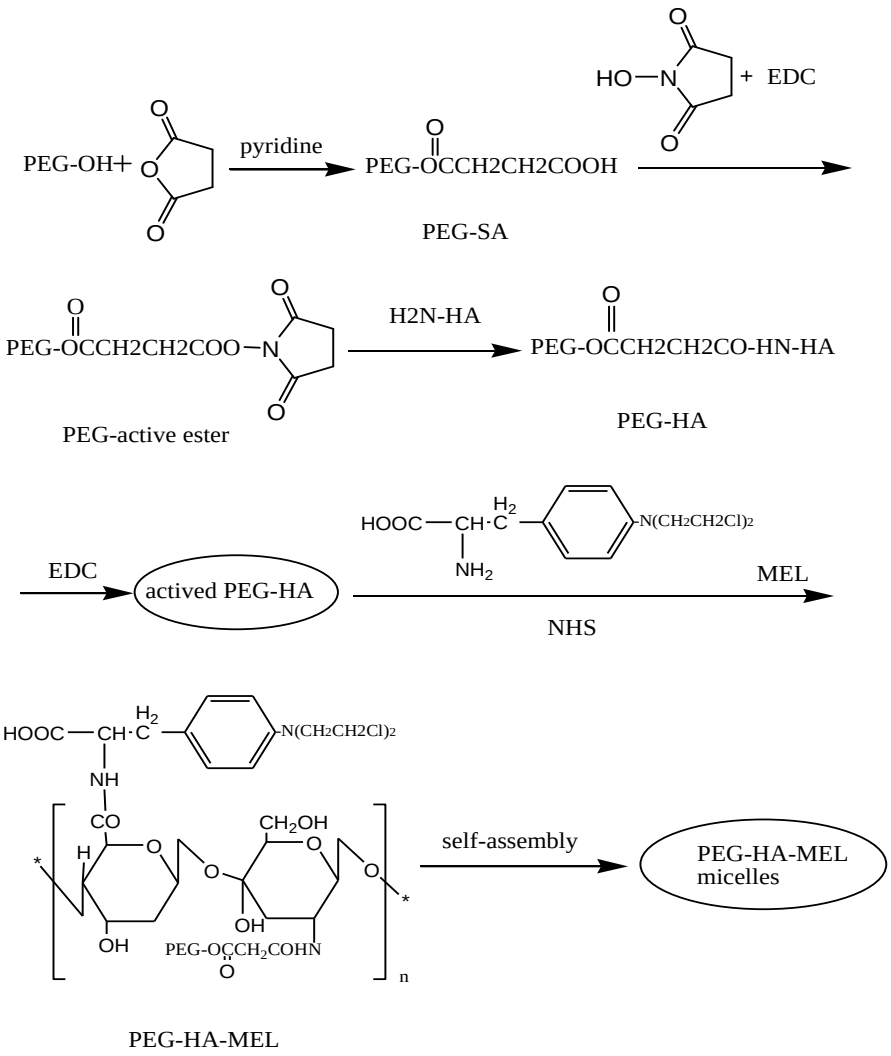

Figure 1: Synthesis process for PEG-HA-MEL and chemical structure of PEG-HA-MEL.

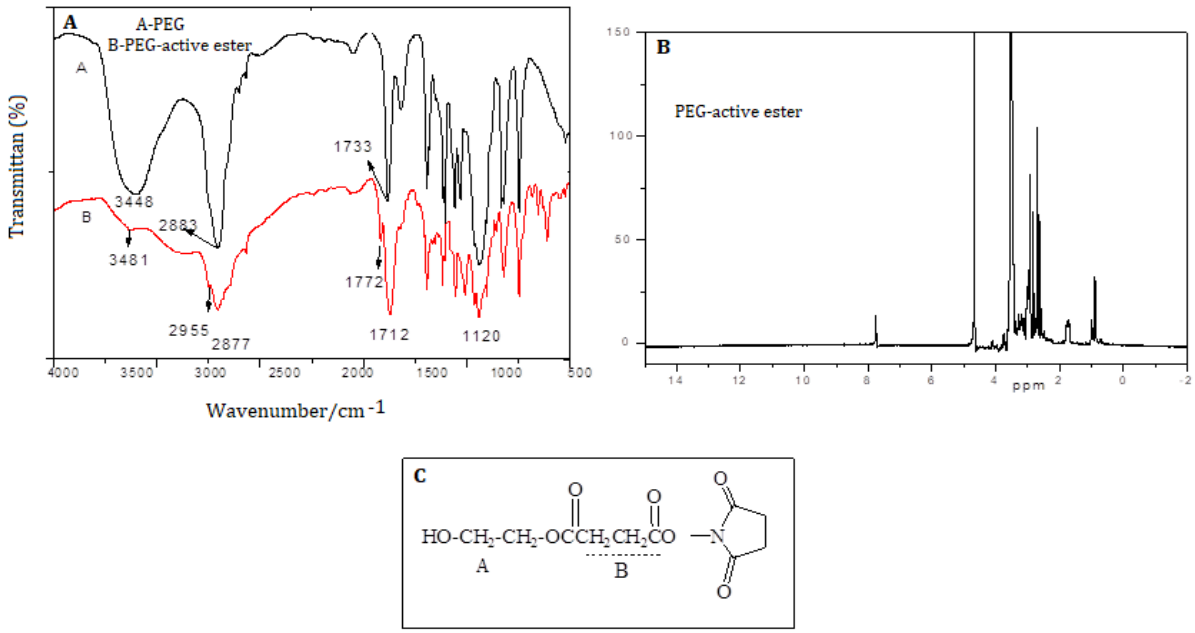

Figure 2: General procedure for preparation of PEG-active ester. A: the FTTR spectra of PEG-active ester. B: The ^{1}H NMR spectra of PEG-active ester; C: conjugation of PEG.

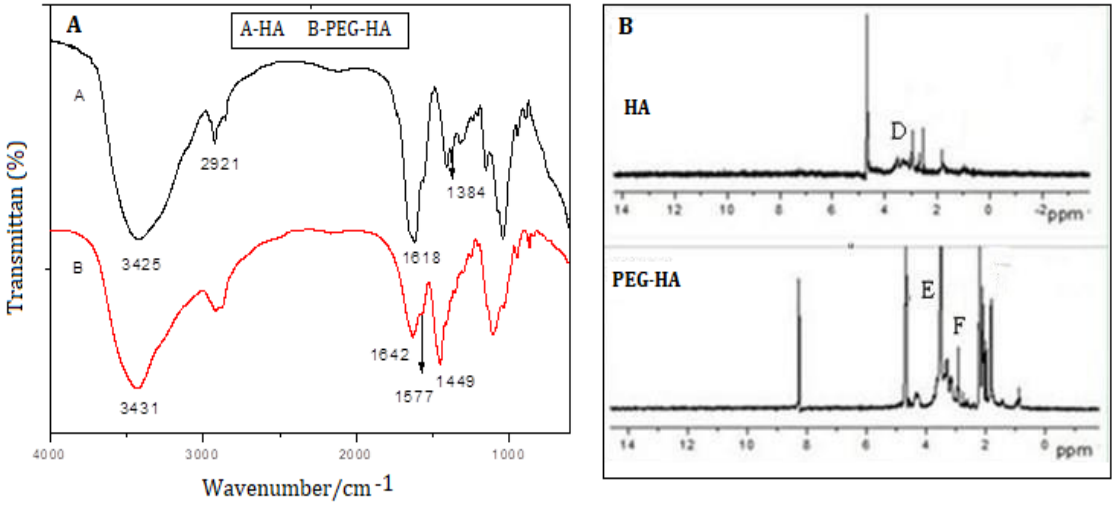

Figure 3. General procedure for preparation of PEGylated HA. A: the FTIR spectra of HA and PEG-HA; B: The[1] H-NMR spectra of HA and PEG-HA.

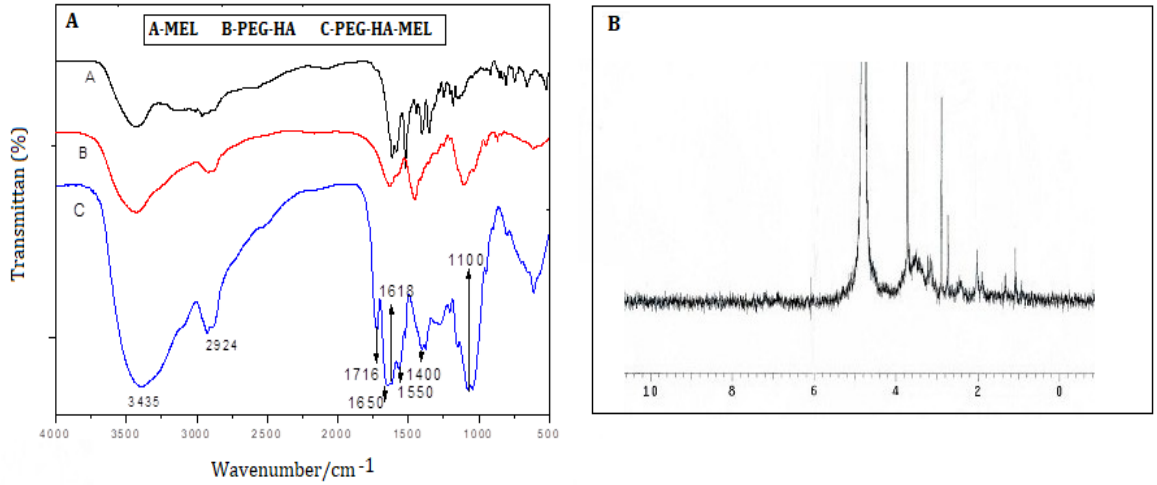

Figure 4: General procedure for preparation of PEG-HA-MEL Micelles. A: the FTTR spectra of MEL. PEG-HA and PEG-HA-MEL; the ^{1}H NMR B: spectum of the micellar solution of PEG-HA-MEL.

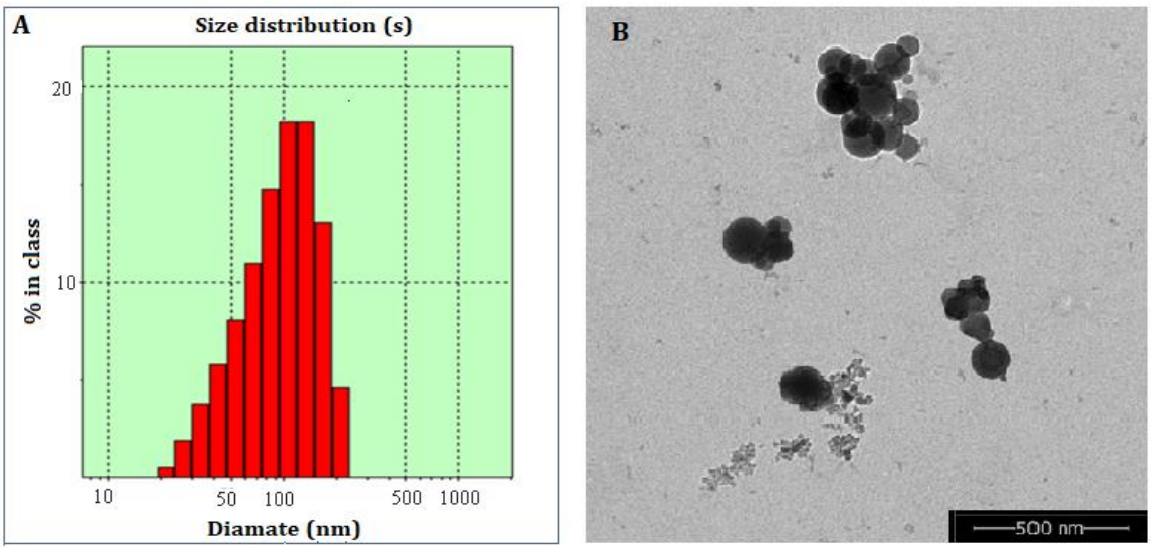

Figure 5: DLS and TEM characterization of PEG-HA-MEL. A: DLS characterization of PEG-HA-MEL. Particle size distribution of PEG-HA-MEL; B: TEM characterization of PEG-HA-MEL, the particle size is around 120nm, and the morphology of micelles is spherical.

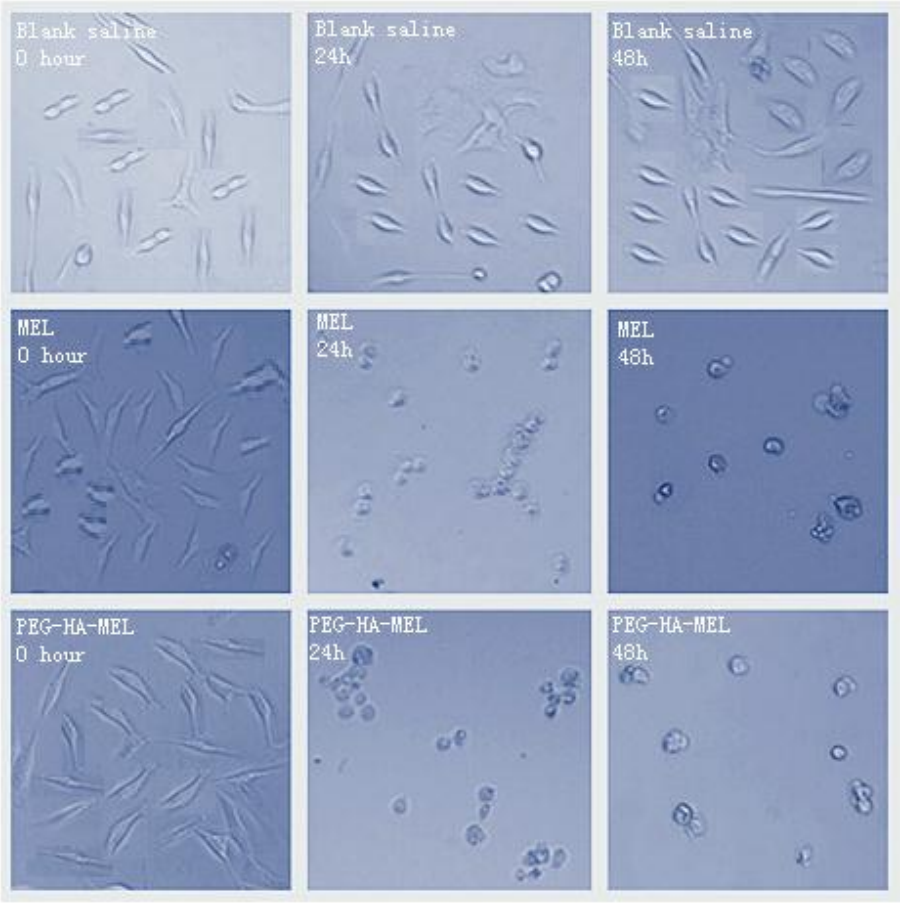

Figure 6: Effects of MEL and PEG-HA-MEL on the morphology of tumor cells.

DISCUSSION

Many studies have shown that there are many proteins that can specifically bind to hyaluronic acid in human and animals, which are mainly distributed on cell matrix and cell membrane. These proteins are called HA-binding proteins (HABP).[19] CD44 is HABP (receptor) which has been studied extensively, and is considered the most important hyaluronic acid receptor on the cell surface.[20] Specifically, the content of CD44 on the surface of tumor cells (such as SKOV3) is many times higher than that of normal cells, which can better combine with HA.[21] Therefore, HA as a carrier can achieve the target effect on tumor cells. HA is selected as a carrier and is suitable for malignant tumor drug delivery system with high expression of CD44 ligand. At the same time, there are some shortcomings of HA such as low water solubility. Therefore, PEG is used to chemically modify it to improve its water solubility and biodegradability, reduce its degradation rate and prolong its retention time in vivo.[22]

This study was to prepare and characterize the new prodrug PEG-HA-MEL to improving the inadequacy of MEL. It has important clinical significance to use chemistry, materials science and life science research result to solve the shortcomings of MEL in order to improve the effective concentration at the tumor site and improve its efficacy.[21,22] HA was modified with activated PEG to prepare PEG-HA to increase its relative molecular mass. PEG-HA was bonded with MEL to obtain targeted drug delivery system and carry out relevant evaluation. It was characterized by nuclear magnetic resonance and infrared spectroscopy.[23,24] Then PEG-HA-MEL was prepared into micelles by dialysis. Particle size distribution and the sample morphology of PEG-HA-MEL was determined by DLS and TEM. It was found that the morphology of micelles is spherical and the particle size is about 120mm.

When the PEG-HA-MEL prodrug enters the body, it binds to cancer cells in a targeted manner. Then, PEG-HA will break with MEL and MEL will free out, thus exerting the drug effect. Cytotoxicity test of the tumor cells treated with PEG-HA-MEL indicated that cells were condensed and rounded, aggregated into clumps, and the number of cells was significantly reduced at 24 hr and 48 hr respectively. PEG-HA-MEL could inhibit tumor growth via regulating CD44 receptor and PEG-HA effect. This is only a preliminary study and theoretical analysis, and further research is needed.

CONCLUSION

The PEG-HA-MEL prodrug was synthesized successfully and characterized by FTIR spectroscopy, ^{1}H-NMR, DLS, and TEM. It can form micelle with the average size about 100-130nm. It was demonstrated that

this natural, biocompatible and biodegradable particles can be used as drug delivery system, and found that this compound play potent inhibitory role on tumor cells, and indicated that the PEG-HA-MEL prodrug could be very useful on release of control targeted drug.

CONFLICT OF INTERESTS: The authors state no conflict of interest.

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