Core cross-linked hyaluronan-styrylpyridinium micelles as a novel carrier for paclitaxel

Youhua Tao, Jing Xu, Mingqing Chen, Huiyu Bai, Xiaoya Liu

1. Introduction

Polymeric micelles formed by self-assembly of amphiphilic block copolymers in aqueous solution have received widespread attention in the past decade (Deng, Li, Yao, He, & Huang, 2010; Guo & Jiang, 2009; Harada & Kataoka, 1999; Jenekhe & Chen, 1998; Liu et al., 2011; Matsumura & Kataoka, 2009; Nishiyama & Kataoka, 2006; O’Reilly, Hawker, & Wooley, 2006; Tao et al., 2009; Wang et al., 2011). Polymeric micelles have a unique core–shell structure which not only enables the system to incorporate poorly soluble drugs but also protects drugs from inactivation in biological media. Due to their small particle size, these systems exhibit many advantages such as targeting ability, long circulation and easy production of effective delivery systems (Chan et al., 2009; Huo, Zhang, Zhou, Zou, & Li, 2011; Li et al., 2011; Torchilin, 2001; Valencia et al., 2011). Polymeric micelles are therefore considered to be an excellent delivery system for hydrophobic anti-cancer drugs. However, one remaining practical challenge for micellar drugs is their inferior in vivo stability (Bae & Yin, 2008). In the past several years, various cross-linking approaches have been adopted to improve micellar stability (O’Reilly et al., 2006; Rijcken, Soga, Hennink, & Nostrum, 2007). The cross-linking of micelles could take place on the hydrophobic shell (Cheng, Khoshdel, & Wooley, 2007), within the hydrophobic core (Li et al., 2009; Talelli et al., 2010), or at the core–shell interface (Xu, Meng, Cheng, & Zhong, 2009). However, there are only a few reports on the development of cross-linked micelles for anticancer drug delivery (Abdullah Al, Lee, Lee, Lee, & Park, 2011; Talelli et al., 2011; Tao, Liu, Chen, Yang, & Liu, 2012; Xiong et al., 2011).

Hyaluronan (also named hyaluronate, hyaluronic acid or HA) is a naturally occurring polysaccharide originally extracted from bovine vitreous humour, rooster combs or umbilical cords (Rinaudo, 2011), and now has been successfully produced on a large scale by Streptococcus bacteria in high purity and good yield (Gao, Du, & Chen, 2006; Ogrodowski, Hokka, & Santana, 2005). It consists of repeating β-(1→4)-d-glucuronic acid β-(1→3)-N-acetyl-d-glucosamine disaccharide units (Chytil, Strand, Christensen, & Pekar, 2011; Chytíl, Pekar, & Chytíl, 2009) (Fig. 1a). Since it is a biopolymer, much current interest focuses on its applications in biomedicine and pharmaceutics (Lee, Lee, & Park, 2008; Lee, Ahn, & Park, 2009; Pitarresi, Pierro, Palumbo, Tripodo, & Giammanna, 2006).

Paclitaxel is known to have highly effective activity against various tumors and has been used clinically in the treatment of metastatic breast cancer, ovarian cancer and several other malignancies. Because the water solubility of PTX is very low, the commercial preparation of PTX is formulated in a solution composed of a 50:50 (v/v) mixture of Cremophor EL (polyoxyylated castor oil) and dehydrated alcohol, which is diluted 5–20 fold in normal saline or dextrose solution (5%) before administration. However, the use of Cremophor EL causes serious side effects such as nephrotoxicity, neurotoxicity and cardiotoxicity. These serious side effects have limited the clinical application...
of Taxol® and caused lots of inconvenience when it was clinical applied. Therefore, it is emergent to find a drug delivery system for paclitaxel without Cremophor EL. In recent years, several drug delivery systems, such as polymeric micelles (Yao, Zhang, Ping, & Yu, 2007; Zhang et al., 2005; Zhang et al., 2008), liposomes and amphiphilic nanoparticles (Bilensoy, Gurkaynak, Dogan, & Hincal, 2008), have been studied to increase solubility of paclitaxel and to minimize the side effects of the formulation, among these new delivery vehicles, polymeric micelles have attracted an increasing interest.

We have previously reported the complex micelles formation between a negatively charged hyaluronan (HA) and an oppositely charged styrylpyridinium (SbQ) in aqueous solution (Xu et al., 2011). Cross-linking of the hydrophobic core via dimerization reaction of the SbQ molecules induced by UV light ultimately produces crosslinked micelles. The purposes of present work were: (1) to encapsulate the hydrophobic anticancer drug paclitaxel into the hydrophobic cores of the cross-linked HA-SbQ micelles, (2) to evaluate the cellular uptake ability of the micelles by bone marrow derived macrophages (BMM) and human glioma U87 cells, (3) to
evaluate the cytotoxicity of both blank micelles and PTX-loaded micelles against BMM and U87 cells and (4) to compare the stability, cellular uptake and cytotoxicity of cross-linked HA-SbQ micelles and un-cross-linked ones and to show the potential of cross-linked HA-SbQ micelles as a novel carrier for anticancer drug.

2. Materials and methods

2.1. Materials

1-Methyl-4-[2-(4-formylphenyl)-ethenyl]-pyridiniummethosulphate (SbQ) was provided by SHOWA KAKO Co. Ltd. (Japan). Sodium hyaluronate (HA), with a viscosity average molecular weight of 22 kDa, was purchased from Bio Chemicals (USA). All other chemicals and solvents were of analytical grade and were used without further purification.

2.2. Preparation of PTX-loaded cross-linked HA-SbQ micelles

HA-SbQ polymeric complex micelles were prepared as described earlier (Xu et al., 2011). Briefly, a HA solution was prepared by dissolving sodium hyaluronate in water, and stirring overnight at room temperature to ensure complete dispersion. Stock solutions of SbQ were prepared by dissolving SbQ in water. The HA solutions were then mixed with SbQ solutions, ensuring the molar ratio of the positively charged SbQ and negatively charged carboxylate groups of HA was equal. To obtain cross-linked particles, the micelle solutions were irradiated under a POWER ARC UV 100 Lamp for 45 min.

PTX-loaded cross-linked HA-SbQ micelles were prepared by dialysis technique (Kim et al., 2006; Wang et al., 2008). Briefly, 0.17 mg PTX dissolved in 5 ml methanol were slowly added to 10 ml crosslinked-HA-SbQ micelles solutions (0.1 mg/ml) under stirring, and then the methanol solvent was removed by dialysis (Millipore dialysis tube, molecular weight cutoff 12–14 kDa, USA) to obtain PTX-loaded cross-linked HA-SbQ micelles. The dust and impurity in the sample solution were removed by passing through a filter (0.45 μm, Millipore). Lissamine rhodamine B 1,2-dihexadecanoyl-sn-glycero-3-phosphoethanolamine, triethylammonium salt (rDHPE; Invitrogen) was used to label cross-linked HA-SbQ micelles and appeared as red fluorescence.

2.3. Characterization of PTX-loaded cross-linked HA-SbQ micelles

Average particle size and size distribution of the micelles were measured by Malvern Zetasizer Naso ZS (Malvern, UK). The shape and surface morphology of the micelles were investigated by Atomic force microscopy (AFM). 100 μl of HA-SbQ micelle solution was placed on a clean mica surface and then air-dried overnight. The image was obtained with a Benyuan CSPM 4000 AFM system.

2.4. Determination of drug loading capability

Drug loading capability of the cross-linked HA-SbQ micelles was determined using high-performance liquid chromatography (HPLC). PTX-loaded micelles solution (50 μl) was transferred into 10 ml volumetric flask and diluted with methanol to the mark. The solution was centrifuged at 10,000 rpm for 10 min, 20 μl of supernatant was injected into the chromatographic system. The HPLC system (LC-2010C, Shimadzu, Japan) was equipped with a Lichrospher C18 column (4.6 mm × 250 mm, 5 μm) with a mobile phase of methanol and water (75:25), the flow rate and column temperature was set at 1 ml/min and 30 °C, respectively. The signals were recorded by UV detector at 227 nm. A calibration line was conducted to determination PTX concentration in the range of 0.5–25 mg/l, and the r²-value of peak area against PTX concentration was at least 0.999. The encapsulation efficiency (EE, %) and the drug loading coefficient (DL, wt.%) were calculated based on the following formulations:

\[ \text{EE (%)} = \frac{\text{weight of PTX in micelles}}{\text{weight of the feeding PTX}} \times 100 \]

\[ \text{DL (wt%)} = \frac{\text{weight of PTX in micelles}}{\text{weight of the feeding micelles and PTX}} \times 100 \]

2.5. PTX releases from cross-linked HA-SbQ micelles in vitro

PTX release behavior was studied in vitro in phosphate buffered saline (PBS) solution. Briefly, the solutions of PTX-loaded cross-linked HA-SbQ micelles were respectively placed into visking dialysis tubing (molecular weight cutoff 12–14 kDa, Millipore, USA) and dialyzed against 50 ml PBS solutions with 1% Tween 80 at 37°C in an air-bath shaker at 50 rpm. Then, 0.5 ml of the release media was collected and replaced with an equal volume of the fresh release media at predefined time intervals. The release amounts of PTX were determined by HPLC method.

2.6. Cell culture

Mice femur bone marrow was dissociated into single cell suspensions. Bone marrow cells and bone marrow derived macrophages (BMM) were cultured with DMEM with fetal bovine serum, 1% glutamine, 10 mg/ml ciprofloxacin, and 500 U/ml macrophage colony stimulating factor (MCSF). Human glioma U87 cells labeled with green fluorescence were obtained from
American Type Culture Collection (ATCC). For the MTT assay, cells were plated on 96-well plates to confluence and were allowed to adhere overnight.

2.7. Fluorescence microscopy

The rDHPE-labeled HA-SbQ micelles exhibited a red fluorescence. Images were captured using a Nikon TE2000-U (Nikon Instruments Inc., Melville, NY) with a swept-field confocal microscope. Fig. 4. PTX release profiles in PBS solutions with 1% Tween 80 at 37 °C. The micelles concentration was 0.1 mg/mL.

Fig. 5. HA-SbQ micelles uptake by BMM cell. (A) Fluorescent microscopy of BMM cell treated with 100 μM rDHPE-labeled cross-linked HA-SbQ micelles. (B) To confirm intracellular drug levels, cell lysates of cultured BMM treated with 100 μM of micelles were collected at specified times and assayed by HPLC.

Fig. 6. HA-SbQ micelles uptake by U87 cell. (A) Fluorescent microscopy of U87 cell treated with 100 μM rDHPE-labeled cross-linked HA-SbQ micelles. (B) To confirm intracellular drug levels, cell lysates of cultured U87 treated with 100 μM of micelles were collected at specified times and assayed by HPLC.
could form nano-sized micelles with a core–shell structure composed of a hydrophobic SbQ core and a hydrophilic HA shell layer (Xu et al., 2011). Cross-linking of the hydrophobic core via dimerization reaction of the SbQ molecules induced by UV light ultimately produces crosslinked micelles (Fig. 1). The anticancer drug, paclitaxel (PTX), is known to have the highly hydrophobic property, so that we hope to use cross-linked HA-SbQ micelles as a carrier for PTX to increase its aqueous solubility. The dialysis method was used to prepare PTX-loaded cross-linked HA-SbQ micelles in this study. PTX dissolved in methanol was first added to the nanoparticle solution, and then was dialyzed against aqueous medium. In the dialysis process, methanol was gradually removed, which resulted that PTX spontaneously transferred from the aqueous medium into the hydrophobic cores of the micelles due to the driving force of hydrophobic interaction. AFM was employed to visualize micelles morphology. AFM image (Fig. 2a) shows that PTX-loaded cross-linked HA-SbQ micelles were still spherical in shape. The size distribution of the micelles was unimodal (Fig. 2b), also indicating the formation of the expected monodispersed micelles.

The physicochemical characterization and drug-loading parameters of PTX-loaded cross-linked HA-SbQ micelles are summarized in Table 1. The amount of PTX (i.e., 10 mg) was kept constant, and concentration of HA-SbQ was varied accordingly (i.e., 20, 30, 50 mg). Formulation of 1/3 (drug/carrirer) was found to be appropriate with particle size of 113 ± 3.5 nm, drug loading coefficient of 30.1 ± 1.9%, and encapsulation ratio of 90.1 ± 1.0%, respectively. The zeta potential of PTX-loaded cross-linked HA-SbQ micelles also showed that the micelle carrier can not only solubilize the poorly water-soluble drugs, but also sustain PTX release. More interestingly, the release of PTX from cross-linked HA-SbQ–PTX micelles was significantly inhibited, compared with those un-cross-linked controls. The suppression in release of PTX was also attributed to the cross-linking, which restricted the mobility of PTX, consequently, its release became slower.

3. Results

3.1. Preparation and characterization of PTX-loaded cross-linked HA-SbQ micelles

Our previous work showed that negatively charged hyaluronan (HA) and an oppositely charged styrylpyridinium species (SbQ) microarray, 488 nm (green) and 568 nm (red) laser excitations, and a 20× objective.

2.8. Cell viability assays

U87 and BMM cell viability was determined using the MTT assay. MTT reduction was measured in U87 and BMM with PTX concentration ranging from 0.001 to 1000 μg/ml for 12 h and then washed out by fresh media. The assay was based on the ability of active mitochondrial dehydrogenase to convert dissolved MTT to water-insoluble purple formazan crystals. To each BMM or U87 of active mitochondrial dehydrogenase to convert dissolved MTT washed out by fresh media. The assay was based on the ability of active mitochondrial dehydrogenase to convert dissolved MTT washed out by fresh media. The assay was based on the ability of active mitochondrial dehydrogenase to convert dissolved MTT washed out by fresh media. Therefore, the less the specific activity of the mitochondrial dehydrogenase is, the less MTT will be reduced to formazan, and the lower the absorbance will be. The absorbance at 490 nm was determined using an ELISA plate reader.

3.2. In vitro drug release study

PTX release behavior from cross-linked HA-SbQ-PTX micelles was studied in vitro in PBS solution with 1% Tween 80 at 37°C. The cumulative PTX release profiles are shown in Fig. 4. Only 35% PTX released from cross-linked HA-SbQ–PTX micelles within 24 h, and approximately 75% PTX was released during 96 h. This result showed that the micelle carrier can not only solubilize the poorly soluble drugs, but also sustain PTX release. More interestingly, the release of PTX from cross-linked HA-SbQ–PTX micelles was significantly inhibited, compared with those un-cross-linked controls. The suppression in release of PTX was also attributed to the cross-linking, which restricted the mobility of PTX, consequently, its release became slower.

3.3. In vitro cellular uptake of micelles

The cellular uptake of HA-SbQ micelles was examined to demonstrate the penetration of the micelles into the cells. The micelles were tested for cell uptake at a constant concentration of 100 μM. As shown in Figs. 5A and 6A, representative images reveal increasing intensity of the fluorescence signal of rDHEPE-labeled cross-linked micelles, demonstrating uptake by BMM and U87 cell.
over the 8-h time period. Cross-linked micelles entered BMM and U87 cell contained varying levels of micelles and increased to 95% by 8 h. HPLC analysis provides quantitative information about the uptake status of micelles. As shown in Figs. 5B and 6B, the uptake of HA-SbQ micelles into BMM and U87 cells was also a time-dependent process which increased with time. The PTX delivered by cross-linked micelles accumulated within the BMM and U87 cells at a faster rate and to larger extent than that by un-cross-linked micelles. Taken together, for PTX-loaded cross-linked HA-SbQ micelles, the smaller size and stable structure may facilitate the cellular uptake, resulting in higher cellular uptake.

3.4. Toxicity

We investigated PTX-loaded micelles toxicity to macrophages and tumor cells. BMM (Fig. 7A) and U87 (Fig. 7B) were treated with varying concentrations of micelles-blank, micelles-PTX and free PTX and cell viability was measured by MTT assay at post treatment day 3. We found no significant toxicity to BMM for micelles-blank, micelles-PTX under experimental dosages, no matter whether micelles were cross-linked or not (Fig. 7A). The results also suggest that HA-SbQ micelles carrier had limited toxicity to macrophages as well as lower bio-toxicity.

Furthermore, it is shown from Fig. 7B that free PTX and PTX-loaded HA-SbQ micelles were specifically toxic to U87 cells at concentrations from 10−2 to 10−6 mg/mL. Chemosensitivity of PTX and PTX-loaded un-cross-linked micelles and PTX-loaded cross-linked micelles was compared. As shown in Fig. 7B, PTX-loaded un-cross-linked micelles, cross-linked micelles displayed increasing cytotoxicity as the concentration increased. PTX-loaded cross-linked HA-SbQ micelles exhibited higher cytotoxicity than un-cross-linked ones and free PTX at identical concentration. The superior cytotoxicity of PTX-loaded cross-linked HA-SbQ micelles might be attributed to higher cellular uptake.

4. Conclusions

In summary, we successfully prepared cross-linked HA-SbQ micelles filled with PTX, and confirmed sustained release for PTX from micelles. Fluorescence microscopy and HPLC studies showed that PTX-loaded cross-linked HA-SbQ micelles had excellent cellular uptake ability by BMM and U87 cells. Cellular uptake of cross-linked HA-SbQ micelles was found to be higher than that of un-cross-linked ones due to smaller size and more stable structure. In vitro cytotoxicity studies also revealed that the PTX-loaded cross-linked micelles were more potent than those of PTX-loaded un-cross-linked micelles and free PTX. Taken together, cross-linked HA-SbQ micelles seem to be a potential drug delivery system of PTX for cancer chemotherapy.

Acknowledgement

This work was supported by National Natural Science Foundation of China (Grant no. 50673038).

References


