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Insight into the role of dual-ligand modification in low molecular weight heparin based nanocarrier for targeted delivery of doxorubicin

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ABSTRACT

Low molecular weight heparin nanoparticles (LMWH) modified by glycyrrhetinic acid (GA) (LMWH-GA) and further decorated by lactobionic acid (LA) (LA-LMWH-GA) were reported as novel hepatocellular carcinoma (HPC)-targeted carriers to overcome multidrug resistance (MDR) of doxorubicin (DOX). The drug-loaded nanoparticles had negative charge of around −25 mV and average size range of 70–170 nm. These nanoparticles performed sustained drug release *in vitro* and prolonged DOX residence time in blood circulation *in vivo*. Compared to free DOX, DOX-loaded nanoparticles demonstrated increased DOX accumulation in drug-resistance HepG2/ADR cells and enhanced *in vitro* therapeutic efficacy. However, DOX/LA-LMWH-GA with dual ligands didn't show higher cellular uptake and cytotoxicity than single GA modified DOX/LMWH-GA, although both GA-mediated and LA-mediated endocytosis were involved in their cell internalization. Uptake pathway inhibition study revealed the less efficacy of DOX/LA-LMWH-GA in cellular level could be attributed to the reduced effect of micropinocytosis and caveolae-mediated endocytosis in cellular uptake. Interestingly, the DOX-loaded nanoparticles developed from lower drug/carrier feeding ratio possessed higher performance in cell internalization and *in vitro* efficacy compared to those developed from higher drug/carrier feeding ratio, which could highlight the role of carrier in drug delivery process.

1. Introduction

Systemic chemotherapy, with the use of various chemotherapeutics, plays a vital role in the treatment of hepatocellular carcinoma (HPC) (Deng et al., 2015). One major problem in the management of HPC patients with continuous chemotherapy is the occurrence of multidrug resistance (MDR), which can obviously reduce the sensitivity of tumor cells to drugs (Chen et al., 2014). The overexpression of ATP binding cassette (ABC) transporters such as *P*-glycoprotein are regarded as a main mechanism of MDR (Jin et al., 2012). Nanomedicine, developed from nanotechnology, provides an effective approach to circumvent MDR by incorporating or conjugating chemotherapeutics to nanocarriers (Yang et al., 2014b). With nano-scaled size, the formulations could accumulate in the tumor tissues *via* passive targeting, and be internalized by cells *via* endocytic pathway which could minimize the effect of *P*-glycoprotein efflux pumps and increase the intracellular drug content (Gao et al., 2012).

Low molecular weight heparin (LMWH), the degradation product of unfractionated heparin, has been extensively applied for the fabrication of drug delivery vehicles owning to its good biocompatibility and biodegradability (Hou et al., 2012). The prevalence of reactive groups in LMWH endows the polysaccharide with various chemical modification strategies such as amination and sulfhydrylation (Yang et al., 2015). By attaching hydrophobic groups to LMWH backbone may confer the polymer with ability to self-assemble into nanostructures in aqueous media and the inner hydrophobic domain can serve as reservoirs for water-insoluble drugs (Li et al., 2014).

To achieve more effective delivery, various ligands which can specifically interact with the targeting sites were introduced into the nanocarriers (Satsangi et al., 2015). Regarding to hepatic targeting, galactose was the most extensively studied targeting moiety which can recognize the asialoglycoprotein receptors overexpressing on the liver hepatocytes' surface. Enhanced cellular uptake and improved therapeutic efficacy were observed after the incorporation of galactose-bearing moiety into the nanoformulations (Ahmed and Narain, 2015). Glycyrrhetinic acid (GA) is an effective hydrophobic liver targeting moiety and could be introduced into hydrophilic chains to form self-assembled nanostructures. For years, hydrophilic polymers such as sulfated

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chitosan, poly(ethylenimine) and hyaluronic acid have been modified with GA and used as self-assembled nano-vehicles for liver targeted delivery (Tian et al., 2012; Wang et al., 2016; Zhang et al., 2013). Hence, we hypothesized that the introduction of GA to LMWH backbone would induce the self-assembly of nanoparticles. With respect to self-assembles containing chemotherapeutic drugs, few studies concerning the effect of drug/carrier ratio in drug formulation on cytotoxicity and cellular uptake are available, and their comparison with free drug in both sensitive and drug-resistant cells remain unclear. Furthermore, synergistic effect of GA-mediated and LA-mediated recognition has been observed in BEL-7402 cells after conjugation of GA and LA to chitosan (Chen et al., 2012). However, whether this dual-ligand modification is suitable for drug-loaded self-assembled nanoparticles in drug-resistant HepG2/ADR cells and how LA modification affect their evaluation in cellular and animal levels need to be clarified.

In the present study, LMWH was firstly modified with GA (LMWH-GA), and then covalently bound with LA to develop a dual-ligand decorated drug carrier (LA-LMWH-GA). Doxorubicin (DOX), a typical chemotherapeutic agent with inherent multidrug resistance, was incorporated into the nanoparticles. Physicochemical characteristics of the blank and drug-loaded nanoparticles were investigated. The cytotoxicity and cellular uptake of DOX solution and DOX-loaded nanoparticles with different drug/carrier composition was performed in HepG2 and HepG2/ADR cells. The *in vitro* drug release, intracellular drug distribution, cell morphological study and *in vivo* pharmacokinetics study of DOX-loaded nanoparticles (DOX/LMWH-GA and DOX/LA-LMWH-GA) were investigated comparably. In addition, the uptake mechanisms investigation of the DOX-loaded nanoparticles was also carried out in HepG2/ADR cells.

2. Material and methods

2.1. Materials

LMWH (Mw ≈ 4000 Da) was a gift kindly donated by National Glycoengineering Research Center of Shandong University (Jinan, China). GA (purity >98% by HPLC) was purchased from Zelang Pharmaceutical Co. (Nanjing, China). Doxorubicin hydrochloride (DOX·HCl), Hoechst 33342, Adipic dihydrazide (ADH), 4-dimethylaminopyridine (DMAP), 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide hydrochloride (EDC), *N*-Hydroxysuccinimide (NHS), triethylamine *N,N,N,N*-tetramethylethylenediamine (TEMED), sulforhodamine B (SRB), trichloroacetic acid, acetic acid and Tris base were obtained from Sigma-Aldrich (China). Other chemical reagents and solvents were of analytical grades and were used as received.

2.2. Synthesis of dual-ligand modified LMWH polymers

The reaction scheme of polymer synthesis is shown in Fig. 1. Aminated LMWH was synthetized based on the coupling reaction between LMWH and ADH as described previously (Li et al., 2014). To obtain glycyrrhetinic acid modified LMWH (LMWH-GA), GA was firstly decorated with a succinic linker to obtain the suc-GA (Tian et al., 2012). At next stage, the carboxylic acid groups of suc-GA (0.1, 0.2, 0.3, 0.4 mol/mol sugar residues of LMWH) were activated by equal amounts (2 equiv./suc-GA) of EDC·HCl and NHS in DMF for 4 h. Then TEMED (2 equiv./suc-GA) were added to the mixture as an acid binding agent and subsequently the reaction mixture was dropped into aminated LMWH solution dissolved in DMF:H2O (3:1, v/v). LMWH was reacted with the activated suc-GA under stirring for 2 days, and then dialyzed against DMF/H2O mixture (3:1, v/v) (3500, MWCO) for 3 days and further against distilled water for another 3 days. Finally, the LMWH-GA polymer was lyophilized.

LA was coupled to LMWH-GA using EDC/NHS catalysis in H_2O, the mixture was allowed to run for 72 h at room temperature, dialyzed and lyophilized to obtain the dual-ligand modified LMWH (LA-LMWH-GA). The structure of the resultant polymer was analyzed by FTIR and ^{1}H NMR. Elemental analysis (Thermo, Italy) was utilized to calculate the LA substitution degree (DS) (the number of LA molecules per 100 sugar residues of LMWH). The self-assembled behaviors and the blood compatibility of polymers were investigated, respectively as the reported method (Du et al., 2014; Jain et al., 2010), and the obtained critical aggregation concentration (CAC) values and hemolysis ratio were used as important parameters to evaluate the polymer conjugates. The apparent molecular weights and molecular weight distributions of LMWH-GA and LA-LMWH-GA was determined by gel permeation chromatography (GPC).

2.3. Nanoparticle preparation

Nanoparticles were prepared by a dialysis method with a minor modification (Guo et al., 2013). Briefly, 10 mg polymer conjugates were suspended in 1 mL water under shaking. After that, 1 mL DMSO was mixed with the conjugates solution and stirred for 12 h. The resultant solution was then dialyzed against water for 48 h to remove DMSO. The nanoparticle solution was further sonicated using a probe-type sonifier, followed by filtration using a 0.45 μm membrane. For drug loading, a certain amount of DOX·HCl and triethylamine (2 equiv./DOX·HCl) were added to DMSO and allowed to react for 24 h to obtain DOX base. Then, the DOX/DMSO solution was dropped to the conjugates solution and stirred for mixing. After dialysis, sonication and filtration described as the blank nanoparticle preparation, the resultant DOX-loaded nanoparticles were stored at 4 °C or lyophilized. The blank nanoparticles and DOX-loaded nanoparticles were characterized by dynamic light scattering (DLS) and transmission electron microscopy (TEM).

2.4. Encapsulation efficiency and in vitro drug release

The amount of incorporated DOX was determined based on the UV absorbance at 481 nm after the extraction of DOX from the nanoparticles using DMSO. The drug loading amount (DL) and entrapment efficiency (EE) were determined as follows:

$$DL\% = \frac{\text{Weight of DOX in nanoparticles}}{\text{Weight of the feeding polymer and DOX}} \times 100\% \quad (1)$$

$$EE\% = \frac{\text{Weight of DOX in nanoparticles}}{\text{Weight of the feeding DOX}} \times 100\% \quad (2)$$

DOX-loaded nanoparticles were sealed in a dialysis bag (Spectrum Laboratories, MWCO 3500) and immersed in 30 mL PBS (0.1 M, pH 7.4 and 5.0, containing 0.2% (w/v) SDS) at 37 °C. At predetermined intervals, 1 mL of solution was withdrawn and replenished with 1 mL fresh medium. DOX concentration was measured by HPLC (Agilent) equipped with a Hypersil-ODS2 column at UV wavelength of 254 nm. The mobile phase was 0.1% SDS:CH_3CN:CH_3OH (50:50:6, v:v:v, containing 0.06‰ phosphoric acid) with a flow rate of 1.0 mL/min.

2.5. Pharmacokinetics study

Twelve male Wistar rats weighting 180–220 g were used. Animals were randomly assigned to three groups, named DOX solution, DOX/LMWH-GA and DOX/LA-LMWH-GA group. All the