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Synthesis, characterization and liver targeting evaluation of self-assembled hyaluronic acid nanoparticles functionalized with glycyrrhetinic acid

Xiaodan Wang, Xiangqin Gu, Huimin Wang, Yujiao Sun, Haiyang Wu, Shirui Mao *

School of Pharmacy, Shenyang Pharmaceutical University, Shenyang 110016, China

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ABSTRACT

Recently, polymeric materials with multiple functions have drawn great attention as the carrier for drug delivery system design. In this study, a series of multifunctional drug delivery carriers, hyaluronic acid (HA)-glycyrrhetinic acid (GA) succinate (HSG) copolymers were synthesized *via* hydroxyl group modification of hyaluronic acid. It was shown that the HSG nanoparticles had sub-spherical shape, and the particle size was in the range of 152.6–260.7 nm depending on GA graft ratio. HSG nanoparticles presented good short term and dilution stability. MTT assay demonstrated all the copolymers presented no significant cytotoxicity. *In vivo* imaging analysis suggested HSG nanoparticles had superior liver targeting efficiency and the liver targeting capacity was GA graft ratio dependent. The accumulation of DiR (a lipophilic, NIR fluorescent cyanine dye)-loaded HSG-6, HSG-12, and HSG-20 nanoparticles in liver was 1.8-, 2.1-, and 2.9-fold higher than that of free DiR. The binding site of GA on HA may influence liver targeting efficiency. These results indicated that HSG copolymers based nanoparticles are potential drug carrier for improved liver targeting.

1. Introduction

Liver cancer is one of the most prevalent fatal diseases and the morbidity is increasing annually (He et al., 2016). The main drawback of traditional chemotherapy is the high cytotoxicity and indiscriminate distribution in various tissues. Therefore, significant efforts have been exerted towards the design and construction of novel nano-drug delivery systems for better therapy of hepatocytes (Wang et al., 2007; Zhang et al., 2012). Self-assembled nanoparticles, based on polymeric amphiphiles, have generated considerable interests as promising liver-targeted drug delivery carriers because they can solubilize various hydrophobic drugs, improve the *in vivo* stability, prolong drug circulation time in the bloodstream, and meanwhile passively target to tumor tissues by the enhanced permeability and retention (EPR) effect (Elsabahy and Wooley, 2012; Tian et al., 2015). However, passive trapping of nanoparticles still cannot guarantee sufficient drug concentration within the cells, which may lead to inefficient cellular uptake (Choi et al., 2010). To overcome the limitations of passive targeting, a variety of liver targeting moieties such as folic acid (Liu et al., 2011), protein (Krishna et al., 2009), and saccharides (Jiang et al., 2009; Jiang et al., 2011), have been installed on the surface of nanoparticles to further improve their therapeutic efficacy by receptor-mediated endocytosis (Deng et al., 2012), but this makes the system even more complicated.

Recently, polymeric materials with multiple functions have drawn great attention as the carrier for nanoparticle drug delivery system design (Arpicco et al., 2014; Mahmoudzadeh et al., 2013). It is highly desirable if carrier and active targeting vector can be combined in one material for system simplicity. Hyaluronic acid (HA), composed of repeating disaccharides of N-acetyl-D-glucosamine and D-glucuronic acid, is a promising constituent of nanoparticles due to its high hydrophilicity and targeting ability (Cho et al., 2012; Zou et al., 2013). HA is found in the extracellular matrix and synovial fluids of most human tissues, thus presenting excellent biological properties such as biocompatibility, biodegradability and low toxicity (Arpicco et al., 2014). It is well known HA-binding receptors such as cluster determinant 44 (CD44) (Arpicco et al., 2014; Tripodo et al., 2015), receptor for hyaluronic acid-mediated motility (RAHMM) (Schiffelers et al., 2004) and lymphatic vessel endothelial receptor-1 (LYVE-1) (Bhang et al., 2009) are overexpressed in malignant cells. HA can specifically bind to cancer cells to increase cellular uptake of drugs by receptor-mediated endocytosis and then enhance targeting therefore therapeutic efficacy (Choi et al., 2010).

Glycyrrhetinic acid (GA) is an active aglycone of glycyrrhizin and possesses several beneficial pharmacological activities, such as anti-inflammatory, antiviral activity and antiulcerative effect (Lu et al., 2008). GA-mediated drug delivery systems have emerged as novel liver targeting platforms since GA molecules could provide hydrophobic

* Corresponding author at: School of Pharmacy, Shenyang Pharmaceutical University, 103 Wenhua Road, 110016 Shenyang, China.
E-mail address: maoshirui@syphu.edu.cn (S. Mao).

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section and liver targeting ligand in combination (Cai et al., 2016; Guo et al., 2013; Tian et al., 2010a). It has been reported that carriers modified with GA have higher accumulation in the liver with superior targeting efficiency to hepatocytes, contributed to the abundant GA receptors on hepatocyte membranes (Tian et al., 2012; Tian et al., 2010b). Furthermore, GA-modified nanoparticles might have the ability to discriminate the normal liver tissue and hepatoma tissue (Tian et al., 2012; Zhang et al., 2012), leading to high therapeutic profile with improved safety.

Therefore, by combining HA and GA in one material *via* appropriate bridge, using HA as the hydrophilic part and GA as the hydrophobic part, not only the nanoparticles can be prepared by self-assembly process, liver targeting can also be enhanced based on the active targeting capacity originated from both HA and GA. However, although GA could be conjugated on HA by modifying its carboxyl groups with the help of different bridging groups such as ethylenediamine (Zhang et al., 2013a), cystamine (Mezghrani et al., 2015), and adipic dihydrazide (Han et al., 2016), it is realized that the carboxyl groups modification might affect the targeting property of HA because the carboxyl groups are the recognition sites for the enzyme and the receptors (Banerji et al., 2007; Schante et al., 2011). Besides, Tian et al. confirmed that the C_3-hydroxyl group in GA has little influence on the targeting ability (Tian et al., 2010a). Thus, in this paper, our hypothesis is that, conjugating GA to HA *via* its hydroxyl group modification might achieve better targeting effect. Moreover, considering that GA presents two functions, as the hydrophobic group and meanwhile as the liver targeting ligand, its content might greatly affect the fate of nanoparticles at different stages. How will the GA graft ratio on HA influence the liver targeting efficiency has not been reported so far.

Thus, in this study, first of all, hyaluronic acid-glycyrrhetinic acid succinate (HSG) with different graft ratios were synthesized and characterized using ^{1}H NMR and FT-IR, the physicochemical properties of the self-assembled nanoparticles were characterized using dynamic light scattering (DLS) and transmission electron microscopy (TEM). The cytotoxicity of HSG nanoparticles against HepG2 cells were evaluated using MTT assay. By using DiR as an indicator, liver targeting efficiency of nanoparticles with different GA graft ratio was investigated using a non-invasive near infrared optical imaging technique in mice. To the best of our knowledge, this is the first time that GA was conjugated to HA *via* hydroxyl group, which may provide better targeting efficiency compared to carboxyl group modification.

2. Materials and methods

2.1. Materials

Hyaluronic acid (HA, 100 kDa) was obtained by oxidative depolymerization (Hokputsa et al., 2003) of HA (200 kDa) supplied by Xian Rongsheng Biotechnology Co. Ltd. (Shanxi, China). Glycyrrhetinic acid (GA) was purchased from Nanjing Zelang Medicine Technology Co. Ltd. (Jiangsu, China). Succinic anhydride was from Tianjin Bodi Chemical Holding Co. Ltd. (Tianjin, China). N,N-dicyclohexyl carbodiimide (DCC) and 4-dimethylaminopyridine (DMAP) were from Shanghai Sinopharm Chemical Reagent Co. Ltd. (Shanghai, China). All other chemicals were of analytical grade and were used without further purification.

2.2. Synthesis and characterization of hyaluronic acid-glycyrrhetinic acid succinate (HSG) copolymers

Hyaluronic acid-glycyrrhetinic acid succinate (HSG) copolymers were synthesized *via* two steps. Firstly, GA (5.0 mmol), succinic anhydride (20.0 mmol) and DMAP (5.0 mmol) were dissolved in 60 mL of dichloromethane (DCM). The mixture was refluxed at 40 °C for 12 h, then the DCM was removed by evaporation. The precipitate was washed with water, then filtered and dried. The white powder of 3-O-hemisuccinate GA (suc-GA) was obtained by recrystallization in ethanol.

Secondly, to activate its carboxyl group, suc-GA was reacted with DCC and DMAP in 20 mL of dimethylformamide (DMF) at 0 °C for 3 h. The molar ratio of DCC:DMAP:suc-GA was 4:1.33:1. Briefly, HA (200 mg) was dissolved in 10 mL of formamide, followed by addition of different amounts of activated suc-GA. After reacting at 40 °C for 36 h, the solution was dialyzed against dimethylsulfoxide (DMSO) for 2 d and distilled water for 3 d using a dialysis membrane (MWCO: 8000–14,000). The dialyzed solution was filtered and lyophilized to obtain the white, sponge-like HSG copolymers.

The structure of HSG was confirmed by ^{1}H NMR and FT-IR. ^{1}H NMR spectra was performed on an AV-600 spectrometer (Bruker, Germany) at room temperature. HA and HSG were dissolved in D_2O and D_2O/DMSO-d_6 (1/4, v/v), respectively, whereas GA and suc-GA were dissolved in $CDCl_3$. FTIR spectra were recorded in the range of 4000 and 400 cm^{-1} with an IFS-55 spectrometer (Bruker, Switzerland) using KBr pellets. The degree of substitution (DS), defined as the number of GA groups per 100 disaccharide units of HA, was determined by UV–Vis spectrophotometer (UV-2000, Unico, Shanghai, China) at 250 nm (Zhang et al., 2013a). The DS was calculated with the following equation:

$$\mathrm{DS}(\%) = \frac{\text{Concentration of GA/Molecular mass of GA}}{\text{(Concentration of HSG-Concentration of GA)/Molecular mass of unit of HA}} \times 100$$

2.3. Determination of critical aggregation concentration (CAC) of HSG

The critical aggregation concentration (CAC) of HSG was determined by fluorescence spectroscopy with pyrene as a probe (Li et al., 2012). Briefly, a known amount of pyrene in acetone was added to a series of 10 mL vials, and acetone was removed by evaporation under nitrogen stream. Then 6 mL of HSG solution in the concentration range from 1×10^{-4} to 1.0 mg/mL, was added to each vial to achieve a final pyrene concentration of 6×10^{-7} M. The solution was sonicated for 30 min and left overnight to equilibrate the pyrene and the nanoparticles. Thereafter, the samples were analyzed by a multimode microplate reader (SpectraMax M3, Molecular Devices, US), with an emission wavelength of 390 nm. The relative excitation fluorescence intensity ratio (I_{338}/I_{334}) was calculated.

2.4. Preparation HSG self-aggregated nanoparticles and DiR-loaded HSG nanoparticles

HSG nanoparticles were prepared by self-assembly in aqueous medium (Yu et al., 2008). Briefly, 10 mg of lyophilized HSG copolymers was dispersed in 10 mL of water (or pH 7.4 PBS to evaluate stability of the nanoparticles) under gentle shaking for 3 h, followed by sonication using a probe-type sonicator (JY92-II, Scientz, Ningbo, China) at 100 W for 10 min under ice bath. Solutions with a concentration of 1 mg/mL were used in the experiment.

The DiR-loaded HSG nanoparticles were prepared by dialysis method (Huo et al., 2012). Briefly, 20 mg of lyophilized HSG was dissolved in 2 mL of formamide and 250 μg of DiR in 250 μL of DMF was added to the above polymer solution. After stirring at room temperature in dark for 24 h, the solution was dialyzed against distilled water for 24 h using a dialysis membrane with a molecular weight cut-off of 8000–14,000. The outer solution was exchanged at 3-h intervals. Subsequently, the dialyzed solution was filtered through a 0.8 μm millipore membrane and then lyophilized.

The amount of DiR in nanoparticles was determined by dissolving the lyophilized nanoparticles in H_2O/DMSO (1/9, v/v) and measuring the absorbance at excitation 748 nm, emission 780 nm using a multimode microplate reader (SpectraMax M3, Molecular Devices, US). The