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Tunable conjugation densities of camptothecin on hyaluronic acid for tumor targeting and reduction-triggered release

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ABSTRACT

Micelles self-assembled from drug-conjugated polymers indicate advantages in alleviating the premature release before reaching the intended site. Hyaluronic acid (HA) is known to specifically bind with a transmembrane glycoprotein CD44, overexpressed in many types of cancerous cells, and can also be served as micelle carriers. However, an excess amount of drug conjugation to HA backbone may be detrimental to the receptor-mediated cellular uptake. Up to now, the effect of conjugation densities of drugs has never been determined on the physical properties and biological performance of resulting micelles. In the current study, camptothecin (CPT) was conjugated on HA through 3,3′-dithiodipropionic acid to self-assemble into reduction-sensitive micelles. The substitution degrees of CPT on HA backbone were tuned from around 4–20%, to clarify the effects on the cellular uptake efficiency and cytotoxicities of micelles, as well as the tumor accumulation and antitumor efficacy. The CPT substitution degree of around 15% on HA resulted in micelles with a higher cytotoxicity to 4T1 cells and achieved a better balance between the cellular uptake and reduction-triggered drug release, compared with other micelles. In contrast to a fast kidney clearance and an even distribution in major organs after intravenous injection of free CPT, the optimized micelles were accumulated in tumors, livers and lungs. The micelle content indicated a significant decrease in livers after 24 h, while that in tumors displayed a significant increase to 4.9% of the injection dose. The tumor accumulation of micelles led to strong tumor suppression with minimal systemic toxicity. The *in situ* tumor inhibition and the accumulation of micelles in liver and lungs inhibited tumor metastasis to these tissues. It demonstrates a feasible strategy to develop drug-HA conjugate micelles with a concise and tunable structure for tumor targeting and reduction-triggered release.

Statement of Significance

Hyaluronic acid (HA) can be served as micelle carriers and targeting ligands to tumor cells. However, the effects of drug conjugation densities on the physical profile and biological performance of resulting micelles have never been investigated. In the current study, camptothecin is conjugated on HA with reduction-sensitive linkers, and the substitution degrees of camptothecin on HA backbone vary from around 4–20%. The micelles with a substitution degree of around 15% achieve a better balance between the cellular uptake and reduction-triggered drug release and a higher cytotoxicity than others. It demonstrates a feasible strategy to develop drug-HA conjugate micelles with a concise and tunable structure for tumor targeting and reduction-triggered release.

1. Introduction

Camptothecin (CPT) is a kind of pentacyclic alkaloid isolated from the bark of a Chinese tree *Camptotheca accuminata*. CPT and its various derivatives have been developed in recent years, displaying various antitumor activities against a wide range of cancers, such as ovarian and lung (topotecan), colon (irinotecan) and brain cancers (gimatecan). Challenges remained in the clinical application of CPT and its derivatives are the lack of water-solubility, lactone ring instability and high adverse drug reaction [1]. It is known that there is a pH-dependent equilibrium for CPT between its lactone and carboxylate form, and the preferentially binding with serum albumin shifts the equilibrium in favor of

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the carboxylate form, which is responsible for severe side effects such as haemorrhagic cystitis, xmyelosuppresion and diarrhea [2]. One of the strategies to increase the water solubility of CPT is the conjugation with water-soluble polymers such as poly(ethylene glycol) (PEG) and poly(L-glutamic acid), which are also supposed to extend the circulatory exposure of CPT [3]. Another strategy to improve the lactone stability of CPT is the encapsulation into liposomes, microspheres, emulsions and solid lipid nanoparticles, which is also supposed to increase the tumor accumulation and decrease the systemic toxicity [4]. Micelles with a core-sheath structure, self-assembled from amphiphilic block copolymers, have received plenty of attentions to enhance the delivery of hydrophobic drugs to tumors [5]. CPT is incorporated into the lipophilic cores of micelles to stabilize the lactone structure via noncovalent and hydrophobic interactions. The hydrophilic sheath is expected to provide a steric stabilization for micelles and alleviate the phagocytosis by the reticuloendothelial system (RES), resulting in a prolonged circulation time and promoted antitumor efficacy. NK012, a micellar formulation of a CPT derivative SN-38 from Nippon Kayaku Co., Ltd, Japan, is in clinical trials now, showing promise in the treatment of malignant gliomas [6].

A passive accumulation into tumor tissues has been identified for the self-assembled micelles by the enhanced permeability and retention (EPR) effect. However, their therapeutic effect may be limited by insufficient cellular uptake by tumor cells, which is dependent on the physicochemical properties of nanocarriers [7]. In addition to the surface charge and size, modified micelles with targeting ligands such as folic acid, transferrin, carbohydrates, peptides, antibody fragments and aptamers, demonstrate an increase in the uptake via receptor-mediated endocytosis [8]. Hyaluronic acid (HA), a naturally occurring polysaccharide, could specifically bind to a transmembrane glycoprotein CD44, which is overexpressed in many types of cancerous cells such as breast, lung, colorectal and melanoma cancer cells. Compared with other types of targeting moieties, HA possesses unique advantages such as non-toxicity, non-immunotoxicity and modification flexibility [9]. HA-paclitaxel and HA-irinotecan conjugates have already been investigated on patients with refractory bladder carcinoma and metastatic colorectal cancer, respectively, showing that therapy based on the HA conjugates is more efficient than that of pristine drugs [10]. In addition, amphiphilic HA derivatives have been prepared by chemical conjugation with lipophilic molecules such as bile acids and cholesterol, or with biodegradable polymers such as poly(caprolactone) and poly(D,L-lactide-co-glycolide) (PLGA), leading to the formation of micelles with compact HA molecules for cellular uptake [11]. Huang et al. developed docetaxel-loaded self-assembled micelles of HA-PLGA block copolymers, showing an enhanced cytotoxicity toward CD44-overexpressing MDA-MB-231 cells [12].

The self-assembled micelles are still in a dynamic state and exhibit a low structural stability in the bloodstream upon intravenous injection. To address the premature release before reaching the intended site, intramicellar cross-linking in the cores or sheaths has been proposed to surmount the blood stability [13]. Tao et al. encapsulated paclitaxel into HA-styrylpyridinium micelles, and the hydrophobic cores were crosslinked via dimerization reaction of styrylpyridinium molecules induced by ultraviolet light, resulting in stabilized micelles under diluted conditions, inhibited premature release and high cellular uptake [14]. To address the low release rate due to the relatively stable linkers, stimuli-responsive release of encapsulated drugs has been designed in response to the intracellular stimuli such as low endosomal pH values, high concentrations of glutathione (GSH) or intracellular enzymes in target cells [15]. Han et al. developed sheath-crosslinked HA-polycaprolactone micelles by the use of 2-(pyridyldithio)-ethylamine conjugated HA and core-crosslinked HA-poly(pyridyl disulfide methacrylate) micelles [16,17], indicating an improved blood stability upon systemic administration, a responsive release to intracellular GSH, and an enhanced antitumor efficacy compared to free drug or conventional micelles. Another strategy to alleviate the premature drug release from micelles is the chemical conjugation of hydrophobic anticancer drugs onto hydrophilic polymers, followed by self-assembling of the drug-conjugated polymers into micelles. Lee et al. prepared micelles from HA-paclitaxel conjugates, exhibited greater cytotoxicity to CD44-overexpressing cells than conventional paclitaxel formulations [18]. In addition to the ability to retain the drug during transport in the blood and tumor tissue, the drug-conjugate micelles must be able to efficiently release the drug once reaching the intracellular target to exert the pharmaceutical action [19]. However, up to now there has been no study regarding the development of stimulus-responsive micelles self-assembled from drug-conjugated HA. Furthermore, an excess amount of drug conjugation to the HA backbone leads to a significant loss of HA characteristics [20], which may be detrimental to the receptor-mediated uptake by cancer cells. However, the effects of CPT conjugation densities on the physical profile and biological performance of resulting micelles have never been investigated.

In the current study, HA conjugates with CPT through 3,3′-dithiodipropionic acid (DTPA) assembled into reduction-sensitive micelles. In contrast to a low level at μM in plasma, GSH is produced intracellularly and maintained at mM levels in the cytosol and subcellular compartments. The concentrations of GSH in the tumor cytosol are several times higher than that in the normal cells [21]. Few attempts have been made to construct drug-conjugated micelles with disulfide linkages, which offer the triggered release of antitumor agents. Zhang et al. constructed CPT-conjugated micelles from copolymers of 4-armed PEG and poly(ε-caprolactone), followed by CPT conjugation through dithiodipropionic acid [22]. In addition, Luo et al. demonstrated that the CPT derivatives released from drug-conjugate micelles remained a higher lactone stability than CPT in the presence of serum albumin and exhibited cytotoxicities similar to that of CPT [23]. In the current study, the substitution degrees of CPT on HA backbone were tuned from around 4–20% to determine the effects on the physical properties of resulting micelles such as drug loading, micelle size and critical micelle concentration (CMC), and biological performance such as cellular uptake and cytotoxicity. The *in vivo* tumor accumulation of drug, antitumor efficacy and metastasis inhibition of the optimal micelles were investigated on tumor-bearing mice.

2. Materials and methods

2.1. Materials

Sodium hyaluronic acid (Mw: 9.8 kDa) was purchased from the Freda Biochem Co., Ltd. (Shandong, China), and CPT was from Knowshine Pharmachemicals Inc. (Shanghai, China). Adipic dihydrazide (ADH), 1-ethyl-3(3-dimethylaminopropyl) carbodiimide (EDC), N,N-dicyclohexylcarbodiimide (DCC), 4-dimethylaminopyridine (DMAP), N-hydroxysuccinimide (NHS), DTPA, and GSH were used as received from Aladdin (Beijing, China). Triton X-100, propidium iodide, trypsin, 3-(4,5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT), pyrene, and dialysis bags were procured from Sigma (St. Louis, MO). Rabbit anti-mouse antibodies of caspase-3 and Ki-67, goat anti-rabbit IgG–horseradish peroxidase (HRP) and 3,3-diaminobenzidine (DAB) developer were purchased from Biosynthesis Biotechnology Co., Ltd. (Beijing, China). All other chemicals were of analytical grade and obtained from Changzheng Regents Company (Chengdu, China), unless otherwise indicated. Dimethyl sulfoxide (DMSO),